The Effects of Interferon Gamma Deficiency on the Autoimmune Inflammation of the Central Nervous System

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Abbreviations

Ab	antibody
Ag	antigen
APC	antigen presenting cell
AT EAE	Adoptive transfer experimental autoimmune encephalomyelitis
BBB	blood brain barrier
BM	bone marrow
BMC	bone marrow chimera
bp	base pair
BSA	bovine serum albumin
CCL	chemokine ligand
CCR	chemokine receptor
CD	cluster of differentiation
CFA	Complete Freund's Adjuvant
CNS	central nervous system
Cre	LoxP site-specific recombinase
CSF	Cerebrospinal Fluid
CTL	cytotoxic T lymphocyte
DAMP	Danger-associated molecular pattern(s)
DC	dendritic cell
DNA	desoxyribonucleic acid
dNTP	desoxynucleotide triphosphate
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylene-diaminetetraacetic acid
EYFP	Enhanced Yellow Fluorescent Protein
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
flox(ed)	LoxP flanked
FoxP3	forkhead box protein 3
GM-CSF	granulocyte/macrophage-colony stimulating factor
HE	Hematoxylin/Eosin stain
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule 1
i.p.	intraperitoneally

i.v.	intravenously
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
IRES	internal ribosome entry site
LFB-PAS	Luxol Fast Blue-Periodic Acid-Schiff stain
LN	lymph node/s
loxP	recognition sequence for Cre
LPS	lipopolysaccharide
mAb	monoclonal antibody
MBP	myelin basic protein
MCP-1	Macrophage chemoattractant protein-1
MHC	major histocompatibility complex
MIP-2	Macrophage inflammatory protein 2
moDC	monocyte-derived dendritic cell
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PAMP	Pathogen-associated molecular pattern(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PNS	peripheral nervous system
PRR	Pattern recognition receptor
Ptx	Pertussis toxin
RAG	recombination activating gene
RNA	ribonucleic acid
RRMS	Relapsing-remitting multiple sclerosis
s.c.	subcutaneously
SLO	secondary lymphoid organs
Taq	polymerase from *Thermus aquaticus*
TCA-3	T cell activation gene 3
TCR	T cell receptor
TE	Tris-EDTA buffer
tg	transgenic
TLR	toll-like receptor
Tregs	regulatory T cells

Aim of the thesis

Clinical studies of MS showed that patients with affected cerebella displayed rapid disease progression and had poor prognosis. At the same time, therapeutic approaches that interfered with the trafficking of activated immune cells had shown promising results.

The main aim of the thesis was to use a mouse model of atypical EAE in the context of IFNγ deficiency and test the hypothesis that there exists a single cell type which, when prevented in sensing IFNγ during the initiating phase of EAE, institutes a series of events that eventually lead to a deep parenchymal infiltration of immune cells into the brain instead to the usual site, the spinal cord, resulting in an extensive tissue destruction at the site.

If proven correct, the implicated cell type and its interactions with the invaded CNS tissue and immune cells were to be investigated in greater detail in an effort to establish the mechanism of action that underlies these decisive early events of EAE.

Summary

Experimental autoimmune encephalomyelitis (EAE) is a robust and valuable animal model of autoimmune inflammation of the central nervous system (CNS). Clinically, in most rodents, it presents itself as an ascending paralysis (rostro-caudal), reflecting inflammation localized predominantly in the spinal cord. Variations from the classical EAE phenotype have also been described (referred to as atypical or nonclassical EAE) and have been shown to occur in certain strain- or antigen combinations and in mice deficient in interferon gamma (IFNγ) or its receptor. The clinical signs observed in atypical EAE reflect increased inflammation of the brain stem and cerebellum whereas the spinal cord is relatively spared. Atypical EAE represents an extension of the classical EAE model since in multiple sclerosis (MS), a human disease often modeled by EAE, inflammatory foci could be found in both the spinal cord and different brain regions.

Although no consensus exists on the mechanisms that lead to the occurrence of atypical EAE, there is an understanding that the inability of the invading T cells to produce IFNγ leads to their particular cytokine and chemokine receptor expression profile that results in their differential homing and a distinct pattern of tissue damage to the CNS. Multiple studies have also suggested that IL17A is indispensable for the occurrence of atypical EAE.

Our work supports a different view, where the inability of a particular cell type (either of the invaded tissue or among the invading cells themselves) to sense IFNγ is the key event that eventually leads to the occurrence of atypical EAE. We show that various mouse strains lacking IFNγ or the functional IFNγ receptor are all susceptible to the induction of atypical EAE and IL17A is completely dispensable for this process. The disrupted IFNγ signaling in neutrophils and a subset of myeloid cells alone is sufficient to drive atypical EAE. The composition of the immune cells infiltrating the CNS is not a determinant of the ensuing clinical manifestation of EAE. The development of atypical EAE in the context of IFNγ deficiency is not mediated by disrupted IFNγ signaling in microglia, as was initially suggested by some authors. Furthermore, absence of IFNγ signaling on vascular endothelium and presumed changes in the expression pattern of its adhesion molecules that result from it have no impact on the capacity of circulating leukocytes to extravasate and reach the CNS during neuroinflammation. Additionally, we were able to show that the inability of the CNS tissue to respond to IFNγ stimulation during neuroinflammation does not

noticeably influence the extent of tissue damage inflicted by the infiltrating immune cells.

Our ability to precisely disrupt IFNγ signaling in a defined cell population which results in the occurrence of atypical EAE allows us to further study the process and its initiating events in unprecedented detail.

Zusammenfassung

Experimentelle autoimmune Enzephalomyelitis (EAE) ist ein häufig verwendetes, anerkanntes und robustes Mausmodell für eine selbstreaktive Entzündungsreaktion im Zentralnervensystem (ZNS). Diese verursacht normalerweise eine rostro-caudal aufsteigende Paralyse, welche durch eine primär im Rückenmark lokalisierte Entzündung verursacht wird. In bestimmten Mausstämmen sowie in Abwesenheit von Interferon gamma (IFNγ) oder dessen Rezeptor und durch bestimmte Antigenkombinationen wurde jedoch eine Abweichung von dem klassischen EAE Erscheinungsbild beschrieben. Die klinischen Kennzeichen dieser atypischen oder nicht-klassischen Form von EAE sind durch eine verstärkte Entzündung des Hirnstamms sowie des Cerebellums bei gleichzeitiger Abwesenheit der Entzündung im Rückenmark geprägt. EAE wird als Mausmodel für die humane Erkrankung Multiple Sklerose verwendet, welche von Entzündungsherden im Rückenmark aber auch in unterschiedlichen Gehirnregionen begleitet ist. Daher stellt das Modell der atypischen EAE eine wertvolle Erweiterung der klassischen Form dar.

Über den genauen Mechanismus der zur Entwicklung von atypischer EAE führt, wurde bislang kein abschließender Konsens gefunden. Es besteht die Annahme, dass infiltrierende T Zellen welche einen Mangel in der IFNγ Produktion haben, zu einem typischen Expressionsprofil von Zytokinen und Chemokinrezeptoren führen welches die Rekrutierung und das Muster der Gewebsschädigung im ZNS beeinflusst. Zusätzlich haben verschiedene Studien IL17A als unabdingbar für das Auftreten von atypischer EAE beschrieben.

Die vorliegende Arbeit argumentiert zugunsten einer neuen Sichtweise, wobei der Defekt eines bestimmten Zelltyps (entweder von dem Zielgewebe oder von den eindringen Zellen selbst) IFNγ zu detektieren und darauf zu reagieren als Schlüsselevent für die Entwicklung von atypischer EAE definiert wird. Wir konnten zeigen, dass verschiedene Mausstämme, die Defekte in IFNγ oder dem IFNγ Rezeptor besitzen, atypische EAE entwickeln und IL17A für diesen Prozess nicht notwendig ist. Spezifische Elimination des IFNγ Signalweges in Neutrophilen und einer Subpopulation von myeloiden Zellen war hingegen ausreichend um die Entwicklung atypischer EAE zu initiieren. Des weiteren zeigen unsere Beobachtungen, dass die Zusammensetzung der infiltrierenden Immunzellen nicht ausschlaggebend für die nachfolgende klinische Ausprägung von EAE ist. Die Entwicklung von atypischer EAE in Abwesenheit von IFNγ ist

nicht durch den fehlenden IFNγ Signalweg in Mikroglia bestimmt auch wenn dies ursprünglich von einigen Gruppen postuliert wurde. Auch das Fehlen des IFNγ Rezeptors im Gefäßsystem und eine möglicherweise damit verbundene Veränderung der Zusammensetzung der Adhäsionsmoleküle hatte keinen Einfluss auf das Potential der zirkulierenden Leukozyten die Blutgefäße zu verlassen und während der Entzündungsreaktion in das ZNS zu gelangen. Zusätzlich konnten wir ausschließen, dass IFNγ sowie die Reaktion darauf im ZNS Gewebe selbst wichtig ist um die Gewebsschädigung durch infiltrierende Immunzellen zu beeinflussen.

Die Möglichkeit den IFNγ Signalweg gezielt in einer bestimmten Zellpopulation auszuschalten ermöglicht uns einen detaillierten Einblick in den genauen Prozess und in die anfänglichen Vorgänge der Entwicklung atypischer EAE.

Introduction

Immune system

The immune system is a complex network of tissues, cells and molecules that act in concert to protect the multicellular organism from threats exerted by pathogens, environmental toxins and deregulated cells of the organism itself. At the same time, very intricate control mechanisms and elaborate partitioning of the different niches within the organism allow the immune system to distinguish between, for instance, harmful and mutualistic microorganisms or deregulated cells and "unusual" behavior of normal cells (changes during pregnancy). This ability is central to the normal functioning of the immune system.

Although all of its components are continuously active and act coordinately, the immune system of higher vertebrates can be seen as operating at three different levels of defense – physical barriers, innate immune system and adaptive immune system. This kind of classification not only represents the sequences of hurdles a pathogen needs to overcome to establish the infection but also the evolutionary pathway from early immune mechanisms shared by plants and animals to the most advanced and complex immune system of vertebrates (Delves and Roitt, 2000a, 2000b; Rodríguez et al., 2012).

The skin and mucosal surfaces represent physical barriers to infection. The innate immune system is comprised of a conglomeration of soluble factors and cells that detect and respond to infectious agents through binding to relatively nonspecific structures common to many pathogens - PAMPs (Pathogen-associated molecular patterns). The adaptive immune system is comprised of T- and B-lymphocytes that recognize highly specific structures (antigens) on microorganisms via highly diverse membrane receptors that are generated randomly and are uniquely tailored to individual pathogens (Rodríguez et al., 2012).

Innate immune responses to infection are rapid (minutes) whereas adaptive immune responses are delayed (days). Innate immune responses are broadly similar between individuals within a population and are generally considered not to improve upon repeated exposure to infectious agents. Adaptive immune responses differ between individuals and improve upon a second or subsequent encounter with the same antigen.

Innate and adaptive immune responses in vertebrates are highly interdependent and cooperate to counteract the infectious agents.

Barriers against infection

Microorganisms are kept out of the body by the skin, the secretion of mucus, ciliary action, the lavaging action of bactericidal fluids (e.g. tears), gastric acid and microbial antagonism.

Immate immunity

Immune responses are initiated through detection of PAMPs representing non-self or danger-associated molecular patterns (DAMPs) that represent hidden self. Pattern recognition receptor molecules (PRRs), which can be either soluble (humoral) or cell-associated, are used by the immune system to detect the presence of PAMPs or DAMPs. PRR engagement leads to a diversity of responses that are aimed at directly killing or engulfing microorganisms via phagocytosis, and also results in amplification of immune responses through release of a range of molecules such as cytokines and chemokines (Matzinger, 1994).

The main phagocytic cells are polymorphonuclear neutrophils and macrophages. The phagocytic cells use their membrane-localized pattern recognition receptors to recognize and adhere to pathogen-associated molecular patterns on the microbe surface. PRRs include Toll-like, C-type lectin, NOD-like, RIG-like and scavenger receptors. PRR engagement leads to activation of phagocyte functions and to secretion of a range of cytokines and chemokines, many of which are expressed in an NFκB- and IRF-dependent manner. Organisms adhering to the phagocyte surface activate the engulfment process and are taken inside the cell where they fuse with cytoplasmic granules. An array of microbicidal mechanisms is, then, available to deactivate the endocytosed organism: the conversion of molecular oxygen to reactive oxygen intermediates, the synthesis of nitric oxide and the release of multiple oxygen-independent factors from the granules. Adherence to PRRs on dendritic cells initiates adaptive immune processes.

The complement system, a multicomponent-triggered enzyme cascade, is used to attract phagocytic cells to the microbes and engulf them. Complement activation also leads to a formation of membrane attack complex (MAC) that perforates microorganisms.

In what is known as the alternative complement pathway, the most abundant component, C3, is split by a convertase enzyme formed from its own cleavage product C3b and factor B and stabilized against breakdown caused by factors H and I, through association with the microbial surface. As it is formed, C3b becomes linked covalently to the microorganism and acts as an opsonin. The

next component, C5, is activated yielding a small peptide, C5a; the residual C5b binds to the surface and assembles the terminal components C6–9 into a membrane attack complex which is freely permeable to solutes and can lead to osmotic lysis. C5a is a potent chemotactic agent for neutrophils and greatly increases capillary permeability. C3a and C5a act on mast cells causing the release of further mediators, such as histamine, leukotriene B_4 and tumor necrosis factor (TNF), with effects on capillary permeability and adhesiveness, and neutrophil chemotaxis. This series of events leads to inflammation.

Inflammation is the term used to describe the events that surround an immune response and includes local swelling (due to recruitment of phagocytes and plasma proteins from blood), redness, pain and temperature elevation. Following the activation of complement with the ensuing attraction and stimulation of neutrophils, the activated phagocytes bind to the C3b-coated microbes by their surface C3b receptors and may then ingest them. The influx of polymorphonuclear cells and the increase in vascular permeability constitute the potent antimicrobial **acute inflammatory response**. Inflammation can also be initiated by tissue macrophages when signaling by bacterial toxins, C5a- or iC3b-coated bacteria adhering to surface complement receptors causes release of neutrophil chemotactic and activating factors.

In additiona to a plethora of membrane-bound PRRs, there exists also a multitude of soluble pattern recognition molecules belonging to several protein families (e.g. pentraxins, collectins, ficolins). Mechanisms of action common to these soluble PRRs upon binding their targets include: opsonization, complement activation, enhanced phagocytic uptake and agglutination (Bottazzi et al., 2010).

For instance, the synthesis of acute phase proteins, such as C-reactive and mannose-binding proteins, is greatly augmented by infection. Mannose-binding lectin generates a complement pathway that is distinct from the alternative pathway in its early reactions. It is a member of the collectin family that includes conglutinin and surfactants SP-A and SP-D, notable for their ability to distinguish microbial from “self” surface carbohydrate groups by their pattern recognition molecules.

Natural killer (NK) cells are innate effector lymphocytes necessary for defence against stressed, microbe-infected, or malignant cells. NK cells kill target cells by either of two major mechanisms that require direct contact between NK cells and target cells. In the first pathway, cytoplasmic granule toxins, predominantly a membrane-disrupting protein perforin and a family of

structurally related serine proteases (granzymes) with various substrate specificities, are secreted by exocytosis and together induce apoptosis of the target cell. The granule-exocytosis pathway potently activates cell-death mechanisms that operate through the activation of apoptotic cysteine proteases (caspases), but can also cause cell death in the absence of activated caspases. The second pathway involves the engagement of death receptors (e.g. Fas/CD95) on target cells by their cognate ligands (e.g. FasL) on NK cells, resulting in classical caspase-dependent apoptosis - a coordinated internal dismantling of critical cellular structures, resulting in a cell death (Smyth et al., 2005).

Large infectious agents that are physically too big to be readily phagocytosed by macrophages and neutrophils are "handled" by eosinophils or basophils. They harbor specific cytoplasmic granules that contain a series of cationic toxins able to kill many targets, including helminths, protozoa, bacteria, and other cells. In bronchial asthma, considerable evidence exists that the eosinophil releases granule proteins, especially the major basic protein (MBP), which in turn mediate tissue abnormalities (Gleich et al., 1993).

Dendritic cells (DCs) provide a conduit between the innate and adaptive immune system by presenting antigen to T-lymphocytes within secondary lymphoid tissues and peripheral tissues. Like macrophages and neutrophils, DCs are considered professional phagocytes. Even if the three cell types phagocytose parasites, bacteria, cell debris, or even intact cells very efficiently, the functional outcomes of the phagocytic event are quite different. Macrophages and neutrophils scavenge and destroy phagocytosed particles, a critical step in innate immunity. DCs, in contrast, have developed means to 'preserve' useful information from the ingested particles that serve to initiate adaptive immune responses. Thus, both phagosomal degradation and acidification are much lower in DCs than in macrophages or neutrophils. Reduced degradation results in the conservation of antigenic peptides and in their increased presentation on major histocompatibility complex class I and II molecules (Signal 1). At the same time they also provide co-stimulatory signals via B7 family ligands (signal 2). Both signals are required for efficient T-cell activation. PAMP-mediated stimulation of DCs triggers their maturation (i.e. the ability to efficiently present antigen and provide co-stimulation) and promotes their migration to lymph nodes (Savina and Amigorena, 2007).

Other cell types of the innate immune system sharing features with T cells are the NKT and γδT cells. NKT cells develop in the thymus, but their T cell

receptor (TCR) is invariant and recognizes glycolipids presented by non-variant CD1d molecule. Due to the rearranging of their TCR genes and the potential to develop a memory phenotype, γδT cells more closely resemble the cells of the adaptive immune system and provide an additional "brifge" between the two (Born et al., 2006).

Monocytes stem from haematopoietic stem cell precursors in the bone marrow and, once released into the blood stream, migrate rapidly to sites of infection and differentiate into macrophages and DC.

Adaptive immunity

The adaptive arm of the immune system implements the power of random somatic rearrangement and somatic hypermutation to generate almost unlimited antigen recognition potential through a cellular system that grants the organism long lasting protection (immunological memory). Lymphocytes, which are divided into B and T cells, are its cellular constituents. B cells are of hematopoietic origin and are generated in the bone marrow where they differentiate into mature resting B cells. T cell precursors also originate in the bone marrow but migrate into the thymus to differentiate into T cells under the guidance of the local stroma. Each clone of B and T cells carries a unique receptor which enables it to detect foreign antigen. B cells and T cells were discovered around the same time following a hypothesized cellular division of those that produce antibodies and originate from the bursa fabricii in birds (place of B cell haematopoiesis in birds; origin of the term B cell) and those responsible for a delayed hypersensitivity (DTH) response and are thymus derived (COOPER et al., 1965).

B cells provide the body's humoral immunity through the production of antigen specific antibodies. Antibodies are soluble B cell receptors (BCRs) that B cells secrete in response to immunogenic stimulation. Antibodies/BCRs consist of a highly diverse part which is randomly assembled by an error-prone genetic rearrangement of the V(D)J loci and a constant region (Fc) which defines the isotype of the antibody. In mice and humans five classes of antibodies exist: IgM, IgD, IgG, IgA and IgE. The isotype defines the function and localisation of the antibody. The induction of a B cell response culminates in a germinal centre reaction in secondary lymphoid organs leading to the immediate generation of antibody producing short lived plasma blasts and affinity matured (product of somatic hypermutation), class switched, long-lived plasma cells (memory B cells) (LeBien and Tedder, 2008). Secreted antibodies have several roles in immunity:

(a) Neutralisation of antigen structures on the surface of pathogens. (b) Opsonisation of pathogens, which directs killing of the pathogen via phagocytosis or the complement system. (c) Marking pathogens for subsequent Fc-receptor mediated effector functions of other leukocytes.

T cell constitute a cellular "branch" of adaptive immunity. Unlike B cells, T cells do not recognize native structures but only peptide antigens presented in the context of MHC molecules by APCs. Their development occurs in the thymus from undifferentiated T cell precursors, thymocytes. Generation of the TCR subunits also involves genomic rearrangement of V(D)J elements and the variability is further increased by a random insertion of nucleotides at most joining sites (N-regions). The TCR is a heterodimer consisting of two transmembrane proteins, either an alpha and beta- or a gamma and delta chain. Either expression determines the lineage fate (von Boehmer and Waldmann, 2010).

The TCR specificity is unpredictable and has potential binding capabilities towards any possible antigen, including self antigens. The thymic positive and negative selection processes eliminate those T cell clones from the T cell repertoire that do not bind (MHC restriction) or bind strongly to self-peptide:MHC complex (spMHC) (recessive/central tolerance). Most of the selection takes place in the medullary region of the thymus where peripheral self-antigens are presented on MHC by immigrating DCs (Bonasio and von Andrian, 2006) and/or medullary epithelial cells by AIRE-dependent ectopic gene expression (Anderson, 2002). The weak interaction between the TCR and spMHC that is necessary to pass positive selection is usually not sufficient to elicit an autoimmune response but acts as a survival signal that is crucial in T cell homeostasis. Besides the weak TCR signalling common γ chain (γc) cytokines (IL-2, IL-7 and IL-15) and their receptors on the T cells regulate T cell survival and expansion (Surh and Sprent, 2008).

Two major subtypes of T cells can be distinguished based on their MHC restriction. $CD4^+$ T cells are MHC class II restricted and recognize antigens presented in the context of MHC class II molecule. CD8+ T cells, also known as cytotoxic lymphocytes (CTL), are MHC I restricted and recognize antigens presented in the context of the ubiquitously expressed MHC class I molecule.

CD4+ T cells that leave the thymus can be divided into 3 distinct lineages: T helper cells (Th cells), natural regulatory cells and natural killer T cells (NKT cells). T helper cells are the central cell type in adaptive immunity. They are

behooved to (a) help B cells produce antibodies, (b) regulate CTL activation, (c) recruit granulocytes to the site of inflammation, (d) stimulate anti-microbial activity by macrophages and (e) orchestrate the overall cellular immune response by the secretion of various cytokines. Loss, misguidance or uncontrolled autoreactivity of Th cells can have a detrimental effect on the organism leading to immunodeficiency, allergy or autoimmunity respectively.

Cytotoxic T cells, if properly primed, home into peripheral tissue and are activated by the foreign peptide:MHC complex on the surface of a cell and in turn start their cytotoxic response which destroys the target cell and contributes to shaping the cytokine milieu at the site of infection. Due to their destructive capacity their priming has to be tightly controlled. A CTL can only become active when a CD4+ T cell counterpart of the CTL is activated and subsequently licenses a DC that cross-presents their cognate antigen on MHC I. Only then the DC can give sufficient costimulatory signal to prime the CTL.

Nervous system

The nervous system is possibly the most complex body system of all, whose purpose is to transmit signals between different parts of the body, coordinate voluntary and involuntary actions of the organism, bridge the sensory structures and other organic systems that respond to them and store memories and similar-level information being generated in the organism's interaction with the environment. The nervous system receives each minute literally millions of bits of information from the different sensory nerves and sensory organs and then integrates all these to determine responses to be made by the body. In many types of organisms, the nervous system consists of two main parts – central nervous system (brain and spinal cord) and peripheral nervous system (nerve fibers). In terms of composition, the nervous system consists of neurons and glial cells.

The basic structure of neurons resembles that of other cells. Thus, each nerve cell has a cell body containing a nucleus, endoplasmic reticulum, ribosomes, Golgi apparatus, mitochondria, and other organelles that are essential to the function of all cells. A particularly noteworthy morphological feature of most nerve cells is the elaborate branching of the dendrites (also called dendritic branches or dendritic processes) that arise from the neuronal cell body. The spectrum of neuronal geometries ranges from a small minority of cells that lack dendrites altogether to neurons with dendritic branches that rival the complexity

of a mature tree. The number of inputs that a particular neuron receives depends on the complexity of its dendritic tree. Nerve cells that lack dendrites are innervated by just one or a few other nerve cells, whereas those with increasingly elaborate dendrites are innervated by a commensurately larger number of other neurons (Purves et al., 2001a). The human central nervous system contains more than 100 billion neurons. Incoming signals enter the neuron through synapses located mostly on the neuronal dendrites, but also on the cell body. For different types of neurons, there may be only a few hundred or as many as 200,000 such synaptic connections from input fibers. Conversely, the output signal travels by way of a single axon leaving the neuron. Then, this axon has many separate branches to other parts of the nervous system or peripheral body. A special feature of most synapses is that the signal normally passes only in the forward direction (from the axon of a preceding neuron to dendrites on cell membranes of subsequent neurons). This forces the signal to travel in required directions for performing specific nervous functions (Guyton, 2006).

Neuroglial cells—usually referred to simply as glial cells or glia—are quite different from nerve cells. The major distinction is that glia do not participate directly in synaptic interactions and electrical signaling, although their supportive functions help define synaptic contacts and maintain the signaling abilities of neurons. Glia are more numerous than nerve cells in the brain, outnumbering them by a ratio of perhaps 3 to 1. Although glial cells also have complex processes extending from their cell bodies, they are generally smaller than neurons, and they lack axons and dendrites. The term *glia* (from the Greek word meaning "glue") reflects the nineteenth-century presumption that these cells held the nervous system together in some way. The word has survived, despite the lack of any evidence that binding nerve cells together is among the many functions of glial cells. Glial roles that *are* well-established include maintaining the ionic milieu of nerve cells, modulating the rate of nerve signal propagation, modulating synaptic action by controlling the uptake of neurotransmitters, providing a scaffold for some aspects of neural development, and aiding in (or preventing, in some instances) recovery from neural injury.

There are three types of glial cells in the mature central nervous system: astrocytes, oligodendrocytes, and microglial cells.

Astrocytes, which are restricted to the brain and spinal cord, have elaborate local processes that give these cells a star-like appearance (hence the

prefix "astro"). The major function of astrocytes is to maintain, in a variety of ways, an appropriate chemical environment for neuronal signaling.

Oligodendrocytes, which are also restricted to the central nervous system, lay down a laminated, lipid-rich wrapping called myelin around some, but not all, axons. Myelin has important effects on the speed of action potential conduction. In the peripheral nervous system, the cells that elaborate myelin are called Schwann cells.

Microglial cells are smaller cells derived from hematopoietic stem cells. They share many properties with tissue macrophages, and are primarily scavenger cells that remove cellular debris from sites of injury or normal cell turnover. Indeed, some neurobiologists prefer to categorize microglia as a type of macrophage. Following brain damage, the number of microglia at the site of injury increases dramatically. Some of these cells proliferate from microglia resident in the brain, while others come from macrophages that migrate to the injured area from the circulation (Purves et al., 2001b).

Three major levels of the central nervous system have specific functional characteristics: (a) the spinal cord level, (b) the lower brain or subcortical level, and the (c) higher brain or cortical level.

We often think of the spinal cord as being only a conduit for signals from the periphery of the body to the brain, or in the opposite direction from the brain back to the body. This is far from the truth. Even after the spinal cord has been cut in the high neck region, many highly organized spinal cord functions still occur. For instance, neuronal circuits in the cord can cause (a) walking movements, (b) reflexes that withdraw portions of the body from painful objects, (c) reflexes that stiffen the legs to support the body against gravity, and (d) reflexes that control local blood vessels, gastrointestinal movements, or urinary excretion. In fact, the upper levels of the nervous system often operate not by sending signals directly to the periphery of the body but by sending signals to the control centers of the cord, simply "commanding" the cord centers to perform their functions.

Many, if not most, of what we call subconscious activities of the body are controlled in the lower areas of the brain—in the medulla, pons, mesencephalon, hypothalamus, thalamus, cerebellum, and basal ganglia. For instance, subconscious control of arterial pressure and respiration is achieved mainly in the medulla and pons. Control of equilibrium is a combined function of the older portions of the cerebellum and the reticular substance of the medulla, pons, and

mesencephalon. Feeding reflexes, such as salivation and licking of the lips in response to the taste of food, are controlled by areas in the medulla, pons, mesencephalon, amygdala, and hypothalamus. And many emotional patterns, such as anger, excitement, sexual response, reaction to pain, and reaction to pleasure, can still occur after destruction of much of the cerebral cortex.

After the preceding account of the many nervous system functions that occur at the cord and lower brain levels, one may ask, what is left for the cerebral cortex to do? The answer to this is complex, but it begins with the fact that the cerebral cortex is an extremely large memory storehouse. The cortex never functions alone but always in association with lower centers of the nervous system. Without the cerebral cortex, the functions of the lower brain centers are often imprecise. The vast storehouse of cortical information usually converts these functions to determinative and precise operations. Finally, the cerebral cortex is essential for most of our thought processes, but it cannot function by itself. In fact, it is the lower brain centers, not the cortex, that initiate wakefulness in the cerebral cortex, thus opening its bank of memories to the thinking machinery of the brain. Thus, each portion of the nervous system performs specific functions. But it is the cortex that opens a world of stored information for use by the mind (Guyton, 2006).

Immunity and the central nervous system

In terms of its immunological status, central nervous system has historically been seen as immunologically privileged site - it was believed that brain does not reject foreign tissue grafts, is devoid of classical APCs, lacks constitutive MHC I and II expression on parenchymal cells and has no lymphatic vessels. However, according to the modern understanding of the immunity of the CNS, this "immune privilege" is seen more as an "immune specialization", since common hallmarks of tissue inflammatory response, like edema, would be detrimental for the delicate network of cells that make the CNS (Engelhardt and Ransohoff, 2005).

Generally, rather strong blood-tissue barriers, low permeability to hydrophilic compounds and carrier-mediated transport systems act as a protection of "privileged" site. Functionally insignificant levels of complement reduce the threat of acute inflammatory reactions and unusually high concentrations of immunosuppressive cytokines, such as IL-10 and TGFβ act as a safeguard against potential uncontrolled immune cell activity. Immune privilege can also be maintained by Fas (CD95)-induced apoptosis of autoaggressive cells.

Tolerance and autoimmunity

An autoimmune disease is a medical condition that occurs when the immune system inflicts damage to the organism's own tissues or cells. The potential for developing autoimmunity is inherent in a way adaptive immunity develops - the randomness of the process can also produce BCRs/TCRs specific for self-antigens. However, this doesn't happen most of the time and the concept is called tolerance towards self and functions on two levels: (a) central and (b) peripheral tolerance. Central tolerance works by eliminating self-reactive T cells that emerge during the thymic development. The importance of central tolerance is shown by deficiency of the autoimmune regulator Aire in humans and mice, which leads to a multiorgan autoimmune disease called the Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Cetani et al., 2001). Peripheral tolerance is the mechanism by which mature T cells that recognize self antigen in the periphery (e.g. because they escaped negative selection or their antigen was not presented in the thymus) are rendered innocuous. This is achieved by anergy, suppression through Tregs or deletion.

There are around 80 different autoimmune diseases recognized so far and they affect approximately 5% of the population in Western countries, with a higher prevalence in females. Based on their restriction, they can be categorized into systemic (e.g. systemic lupus erythematosus, rheumatoid arthritis) and organ-specific autoimmune diseases (e.g., multiple sclerosis, autoimmune thyroiditis). All of them tend to have both genetic and environmental background. For example, as mentioned, mutations in the *Aire* gene lead do autoimmunity and many other genes have been strongly correlated with certain diseases, so-called risk alleles (DRB1*1501 in MS, for instance) (International Multiple Sclerosis Genetics Consortium et al., 2007). But the fact that healthy people can carry those same alleles suggests that there is also an environmental factors that likely play a role. Viral and bacterial infections can contribute in two different ways: A local immune response can be mediated by activation of costimulatory molecules and cytokine production which in turn can break tolerance and result in a bystander activation unspecific for the pathogen but directed against a self antigen. The second possibility is molecular mimicry: a pathogen might contain an antigen structurally similar to a self antigen (mimicking the self antigen) and therefore elicit an immune response against self.

MS

Multiple sclerosis (MS) is the most common neurological disorder in young adults in the developed world. It is a complex disease with heterogeneous clinical, pathological and immunological phenotype that might better be described as a syndrome rather than a single disease entity—a concept that has important implications with respect to the development of effective therapeutic strategies. The clinical heterogeneity of multiple sclerosis has been recognized for many years, but it is now apparent that this heterogeneity extends to both the genetics of the disease and the pathological mechanisms involved in lesion formation. Clinically the illness may present itself as a relapsing–remitting disease, or with steady progression of neurological disability. The subsequent course of disease is unpredictable, although most patients with a relapsing–remitting disease will eventually develop secondary progressive disease. Advances in molecular medicine have clearly demonstrated the heterogeneity of multiple sclerosis (Gold et al., 2006; Lassmann et al., 2001).

EAE

Experimental autoimmune encephalomyelitis (also historically referred to as autoimmune allergic encephalitis or encephalomyelitis – EAE) is currently the best characterized animal model of human autoimmune disease. It's origins date back to the 1920s, when Koritschoner and Schweinburg induced spinal cord inflammation in rabbits by inoculation with human spinal cord (Koritschoner and Schweinburg, 1925). In the 1930s, researchers attempted to reproduce the encephalitic complications associated with rabies vaccination by repetitive immunization of rhesus monkeys with CNS tissue (Rivers and Schwentker, 1935; Rivers et al., 1933). In years to come it became clear that EAE can reproduce many of the clinical, neuropathological and immunological aspects of multiple sclerosis (WOLF et al., 1947). However, EAE did not make it into the mainstream research at the beginning since the process of disease induction in laboratory animals was laborious and lengthy (repeated injections over a period of a year). It took almost two decades before the procedure was facilitated through use of newly developed oil-based adjuvants and addition of heat-inactivated mycobacteria tuberculosis to it (Kabat et al., 1951).

Although sometimes criticized for its inadequacy as a model of multiple sclerosis (Sriram and Steiner, 2005) it is a valuable tool for studying basic principles of autoimmune inflammatory processes in the CNS. In most rodents,

EAE manifests itself as an ascending flaccid paralysis in rostro-caudal direction, reflecting the preferential localization of inflammatory foci in the spinal cord. This pattern of clinical features is described as classical EAE. However, in certain combinations of animal strain and immunizing agent, a different set of clinical features was described, thus dubbed atypical EAE (Greer et al., 1996; Muller et al., 2000). In addition, interferon gamma (IFN-γ) deficiency was also found to result in atypical EAE (Wensky et al., 2005; Abromson-Leeman et al., 2004). In atypical EAE the clinical picture is dominated by apparent defects in balance and proprioception. Pathohistological analysis of the CNS in these animals reveals increased inflammation of the brain stem and cerebellum compared to the spinal cord (Muller et al., 2000).

The interferon system

The interferons (IFNs) were originally discovered as agents that interfere with viral replication (Isaacs and Lindenmann, 1957). Initially, they were classified by the secreting cell type but are now classified into type I and type II according to receptor specificity and sequence homology. The type I IFNs are comprised of multiple IFNα subtypes, IFNβ, IFNω and IFNτ, all of which are structurally related and bind to a common heterodimeric receptor (IFNAR, comprised of IFNAR1 and IFNAR2 chains). IFNγ is the sole type II interferon. It is structurally unrelated to type I IFNs, binds to a different receptor, and is encoded by a separate chromosomal locus. The main producers of IFNγ are T cells and NK cells, although some other cell types have also been implicated as potential cellular source (Young, 1996). IFNγ production is controlled by cytokines secreted by APCs, most notably interleukin 12 and IL-18. These cytokines serve as a bridge to link infection with IFNγ production in the innate immune response (Otani et al., 1999). Negative regulators of IFNγ production include IL-4, IL- 10, TGFβ and glucocorticoids. Given the complexity of IFN-γ regulation, it is not surprising that inbred mouse strains vary in their ability to secrete this cytokine; for example, T lymphocytes of C57BL/6 and C3H mice secrete significantly higher amounts of IFNγ compared with the T lymphocytes of BALB/c and B10.D2 mice. IFNγ coordinates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes (Schroder et al., 2004).

Functional IFNγ receptor (IFNGR) is comprised of two ligand-binding IFNGR1 chains associated with two signal-transducing IFNGR2 chains and

associated signaling machinery. IFNGR1 and IFNGR2 chains belong to the class II cytokine receptor family, a class of receptors that bind ligand in the small angle of a V formed by the two Ig-like folds that constitute the extracellular domain (Thoreau et al., 1991). The IFNGR2 chain is generally the limiting factor in IFNγ responsiveness, as the IFNGR1 chain is usually in surplus (Bernabei et al., 2001). The IFNGR2 chain is constitutively expressed, but its expression level may be tightly regulated according to the state of cellular differentiation or activation (Bach et al., 1997). Both IFNGR chains lack intrinsic kinase/phosphatase activity and so must associate with signaling machinery for signal transduction. The IFNGR1 intracellular domain contains binding motifs for the Janus tyrosine kinase (Jak)1 and the latent cytosolic factor, signal transducer and activator of transcription (Stat)1. The intracellular region of IFNGR2 contains a noncontiguous binding motif for recruitment of Jak2 kinase for participation in signal transduction.

Mice without the IFNγ receptor had no overt anomalies, and their immune system appeared to develop normally. However, mutant mice had a defective natural resistance, they had increased susceptibility to infection by *Listeria monocytogenes* and vaccinia virus despite normal cytotoxic and T helper cell responses (Huang et al., 1993).

In addition to recurrent infection, infants with deficient production of IFNγ exhibited decreased neutrophil mobility and NK cell activity, highlighting the importance of IFN-γ in the inflammatory response and immunoregulation (Davies et al., 1982).

Cre-LoxP system

The Cre/loxP recombination system is the most widely used system for cell-type-specific gene inactivation. The core of this system is the Cre recombinase, a protein from bacteriophage P1, which mediates recombination of specific sequences called loxP sites. When two loxP sites are introduced into the genome in the same orientation and the Cre recombinase is expressed, the Cre will recombine the loxP sites and delete the sequence between the sites. In this way the Cre/loxP system can be used to delete genes or critical parts of genes. The system is also widely used for conditional gene activation. In this setting a cassette that is blocking transcription, a so-called ‘‘stop-cassette’’ that is flanked by loxP sites, is introduced between a promoter and the translational start site of

a gene. Thus, the gene is silent under basal conditions but activated upon Cre-mediated removal of the stop-cassette (Sauer, 1993).

Cre fusion proteins have been developed which are inactive in the basal state since they are fused to a modified ligand-binding domain of the estrogen receptor and thus kept in the cytoplasm. Upon binding of tamoxifen, a synthetic steroid, the fusion protein is translocated to the nucleus and the Cre is able to perform the recombination of the loxP sites. Furthermore, in order to restrict gene inactivation in a certain cell-type or tissue, the Cre fusion protein can be expressed under a tissue-type-specific gene promoter (Feil et al., 2009).

Matherials and Methods

Mice and animal experiments

All mice were kept in-house in individually ventilated cages under specific pathogen-free (SPF) conditions. C57BL/6 (wild-type) mice were purchased from Janvier.

IFNγ-KO mice (GKO) were obtained from Jackson Laboratories.

IFNGR1-KO mice were obtained from Jackson Laboratories.

Ifngr2^{tm1Hzi} mice were obtained from Prof. Werner Muller, University of Manchester.

Nestin-Cre mice were obtained from Prof. Max Gassmann, University of Zurich.

VE-Cadherin-Cre mice were obtained from Prof. Cornelia Halin Winter, ETH Zurich.

CX3CR1-CreERt2 mice were obtained from Prof. Steffen Jung, Weizmann Institute of Science.

CD4-Cre mice were obtained from Jackson Laboratories.

LysM-Cre mice were obtained from Jackson Laboratories.

All animal experiments were approved by Swiss Veterinary Office and performed sccording to the appropriate experimental license (55-2009 and 86-2012).

DNA isolation and genotyping

Biopsies were obtained either by ear-punching adult mice, or by toetip-clipping pups before reaching the age of 12 days. Biopsies were incubated in 500 µl tail lysis buffer containing 10 µl Proteinase K for 1-3 hours at 55°C, followed by centrifugation for 5 minutes at 14.000g to pellet undigested tissue. The supernatant was transferred to a fresh tube and 500 µl of isopropanol was added to precipitate the DNA, followed by centrifugation for 10 minutes at 14.000g / 4°C. The supernatant was discarded and the DNA pellets were air-dried for 10-15 minutes and then resuspended in 50-100 µl of TE buffer.

For genotyping, 1 µl of the purified DNA was used as template, together with the appropriate primers and PCR protocols. PCR products were analyzed by running a standard 1-2% agarose Gel in TAE buffer.

Bone marrow chimeric mice.

Bone marrow (BM) donor mice were euthanized using CO_2 and BM cells were isolated by flushing femur, tibia, humerus and hip bones with phosphate-buffered saline (PBS). BM cells were filtered through a 70-μm cell strainer (BD) and cells were washed with PBS. Recipient mice were lethally irradiated with 1100 rads (split dose) and intravenously injected with 10×10^6 BM cells. Engraftment took place over the following 6 weeks of recovery. Mice were examined prior to EAE induction to assess the extent of engraftment of donor-derived leukocytes.

Induction and scoring of EAE.

Experimental autoimmune encephalomyelitis (EAE) was induced as described previously (Gyülvészi et al., 2009). Briefly, mice were immunized subcutaneously in the flanks with a total amount of 200 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK; GenScript) emulsified in CFA (Difco) in a total volume of 200 μl and two intraperitoneal injections of 200 ng Pertussis toxin (Sigma) on day 0 and 2. For immunization of bone marrow chimeric mice same procedure was followed but the usage of pertussis toxin was omitted.

For passive EAE induction, spleen and lymph node cells were harvested from donor mice of the indicated genotype on day 7 post immunization, re-stimulated *in vitro* for two days with 20 μg/ml MOG and 20 ng/ml IL-23 and then i.v. transferred to recipients who were sub-lethally irradiated (550 Rad) one day before the transfer.

Mice were assessed clinically by daily weighing and observation. Classical EAE signs were assessed according to the following criteria: 0, no disease; 1, decreased tail tone or slightly clumsy gait; 2, tail atony and/or moderately clumsy gait and/or poor righting ability; 3, limb weakness; 4, limb paralysis; 5, moribund state.

The severity of atypical EAE was assessed according to the criteria adapted from previous publications (Greer et al., 1996): 0, No detectable signs; 1, Head turning; 2, Pronounced head turning and body leaning to the side; 3, Inability to walk straight; 4, Inability maintain upright posture without support; 5, rolling and spinning in axial direction.

Since different grading scales are used to asses disease progression of classic and atypical EAE, the results are never depicted on the same graph. Sometimes the individual mouse will display combination of atypical and classic

symptoms (limp distal tail and tilted body posture, for instance) which complicates the assignment of the unique score and further supports the approach of depicting disease progression curves as two independent graphs. It sometimes occurs (most often at the disease onset) that an animal displays classic or atypical disease symptom, only to switch to a different group of symptoms the next day. To accommodate these occurrences, the criteria for declaring a strain as displaying atypical disease were defined as minimum of two consecutive days of showing unambiguous atypical symptoms. In our hands, unlike reported elsewhere (Stromnes and Goverman, 2006), the symptoms of atypical EAE in mouse strains with a specific genetic makeup tend to appear in chronological order and are backed up with the progressive weight loss. Sporadic, non-enduring occurrences of atypical EAE symptoms do not show this regular increasing pattern.

Tamoxifen treatment

Mice (starting from 4 weeks of age) were fed with Tamoxifen food pellets (Harlan Teklad CRD TAM400/CreER) containing 400 mg tamoxifen/kg for 3 - 4 weeks. Mice were monitored for their behavior and health status.

Mice were injected i.p. with 2.5μg of tamoxifen (Sigma, T56481G) dissolved in 50μL of corn oil (Sigma, C8267) every second day, total of 4 times.

Leukocyte isolation from the tissues

CNS

Mice are euthanized with CO_2 followed by the transcardial perfusion with PBS through the left ventricle. The animal is decapitated at the level of the shoulders and spinal cord is removed by flushing the spinal column with sterile PBS through the incision at the base of the column. The brain is dissected by opening the top of the scull and removing the brain with metal spatula. Both tissues are homogenized and cell suspension filtered through a 100 mum nylon filter (Fisher). After centrifugation, the pellet is resuspended in 30% isotonic Percoll. The sample is centrifuged at 10000 g for 25 min at 4 °C, without brakes. The myelin fraction forms a thick layer on the surface and is sucked away. The remaining, leukocyte-containing fraction is collected in a new tube, omitting the fraction at the bottom that contains erythrocytes. Collected leukocytes are additionally washed and prepared for staining.

Lung

For lymphocyte isolation from the lung, lungs were cut into small pieces using scissors and incubated in 1 mg/ml collagenase D (Roche) and 0.1 mg/ml DNase (Sigma) in RPMI for approximately 60 minutes at 37°C. Remaining pieces of tissue were homogenized using syringes and 20 gauge needles. The cell suspension was filtered via 70 μm cell strainers and washed with PBS. RBC lysis was performed using 1 ml of RBC lysis buffer and 8 minutes of incubation on ice, followed by a washing step with PBS. The obtained cells were used for further procedures.

Histological analysis

Samples of CNS tissues were perfused with PBS and fixed by immersion in formalin. To visualize the infiltrating immune cells at the site of inflammation, tissue sections were stained with hematoxylin and eosin (H&E). The extent of demylination was assessed based on Luxol fast blue/Periodic acid Schiff (LFB/PAS) staining.

Formalin-fixed samples were embedded in paraffine and co-stained with primary antibody rabbit anti–phospho-STAT1 (detecting only phosphorylated STAT1 at position Tyrosine 701; Cell Signaling Technology) together with either mouse anti-neuronal nuclei NeuN (Neurons, EMD Millipore) or mouse anti-glial fibrillary acidic protein (astrocytes; Dako) or mouse anti-NogoA (oligodendrocytes, mAb11c7 (Oertle et al., 2003)) or rat anti-mouse Mac3 (microglia/macrophages; BD Pharmingen) antibody. Bound primary antibodies were visualized using the appropriate species-specific Cy2- or Cy3-conjugated secondary antibodies (all from Jackson ImmunoResearch Laboratories, Inc.) with DAPI (Sigma-Aldrich) nuclei for fluorescence microscopy. Stained slides were subsequently scanned using the 3D Histech 250 Flash slide scanner at 200-fold magnification to evaluate cellular colocalization of nuclear P-STAT1 signal with aforementioned cell specific marker.

Flow Cytometry

For flow cytometric analysis, the following Abs were used:

Clone	Target	Manufacturer
GK1.5	CD4	BioLegend / BD
M1/70	CD11b	BioLegend

N418	CD11c	BioLegend
30F11	CD45	BioLegend / BD
MEL-14	CD62L	BD
M5/114.15.2	I-A/I-E	BioLegend
Al-21 or HK1.4	Ly6C	BD / eBioscience
1A8	Ly6G	BD
XMG1.2	IFN-γ	BioLegend / BD
TC11-18H10 or 17B7	IL-17	BioLegend / BD
MP1-22E9	GM-CSF	BD
4a	pSTAT1	BD

The cells were acquired and analyzed using the FACSCanto II and LSRFortessa (BD) with FACSDiva software. Cell sorting was carried out using a FACSAria III (BD). Postacquisition analysis was performed using FlowJo software. For all experiments, dead cells were excluded from the analysis using an Aqua Live/Dead fixable staining reagent (Invitrogen) (Perfetto et al., 2010), and doublets were excluded by FSC-Area vs FSC-Height gating.

For intracellular cytokine staining, cells were incubated 3-5 hours in RPMI-1640 containing 10% FCS with PMA (50ng/ml), Ionomycin (500ng/ml) and GolgiPlug (containing Brefeldin A, BD, 1:1000 dilution). Cytofix/Cytoperm (BD) was used according to the manufacturer's instructions, and Perm/Wash Buffer was either obtained from BD or prepared in-house.

Intranuclear staining of phosphorilated STAT1 was performed according to the manufacturer's instructions. Surface epitopes of cells were first stained, followed by 30 minutes stimulation with recombinant mouse IFNγ (Peprotech) at a concentration of 40ng/ml. Cells were fixed with formalin and permeablized with methanol, followed by staining with anti-pSTAT1 mAb (BD)

Pansorbin assay

PANSORBIN cells (Calbiochem) were dissolved in PBS at the concentration of 1μg/50μL and administered intranasaly to anesthetized mice. Animals were euthanized 6h later and lungs were transcardially perfused and harvested for leukocyte isolation.

Statistical analysis

Frequency distribution of two distinct clinical outcomes of EAE (classical vs. atypical) across two experimental groups (tissue-specific disruption of IFNγ signaling vs. intact IFNγ signaling) was tabulated in a contingency table and analyzed for statistical significance using the Fisher's exact test. A null-hypothesis (no relationship between variables) was rejected whenever a p-value was smaller than 0.05.

Buffers and chemicals

Staining buffer:

PBS (phosphate buffered saline, Kantonsapotheke Zurich)
2% FCS
0.005% Sodium Azide

MACS buffer:

PBS
5 g of bovine serum albumin (BSA, Sigma)
4 ml of 0.5M EDTA

Biopsy lysis buffer:

50 ml 1M Tris/HCl pH 7.5
5 ml 0.5M EDTA (AppliChem)
5 ml 20% SDS
20 ml 5M NaCl
ad 500ml with H_2O bidest

Red blood cell lysis buffer:

4.15 g NH_4Cl
0.55 g $KHCO_3$
0.185 g EDTA
ad 500 ml with H_2O bidest, sterile filtration

Homemade Perm/Wash Buffer:

PBS
2% BSA
0.5% Saponin

TAE buffer (50x):

484 g Tris base
14.2 ml acetic acid
200 ml 0.5M EDTA (pH 8.0)
adjust pH to 7.5
ad 2000 ml with H_2O dest

TE buffer:

10mM TrisCl pH 7.5
1mM EDTA (2 ml for 1000 ml of buffer)

Digestion medium:

RPMI (Gibco or PAN)
2% FCS
25mM HEPES

Cell culture medium for *in vitro* culture of lymphocytes:

RPMI 1640 (Gibco)
10% FCS
Penicillin/Streptamycin
L-Glutamin (final conc. 2mM)

Results

Animals lacking IFNγ are susceptible to EAE and display atypical disease symptoms also in the absence of IL-17A

It has been shown previously that neither IFNγ signaling (as in IFNγ-deficient mice) nor IL17A/F signaling (as in IL17RA-deficient mice) are necessary for the development of EAE (Kroenke et al., 2010). However, the same authors postulated that atypical EAE seen in WT mice adoptively transferred with IFNγ-deficient effector T cells is dependent on a functional IL17 receptor, as IL17RA-deficient recipients revert back to the classical EAE disease course upon transfer. Others have suggested that atypical EAE is a feature of a disturbed Th1/Th17 balance and occurs when the Th1/Th17 ratio is more in favor of Th17 cells (Stromnes et al., 2008).

To examine how IFNγ-deficient mice respond to active EAE induction and investigate the role of IL17A during this process, we have generated a novel mouse strain that is deficient in both IFNγ and IL17A (termed DKO - **D**ouble **K**nock**O**ut) and compared the EAE features after active induction to the ones seen in IFNγ- and IL17A-single-deficient mice (termed GKO and IL17A KO, respectively). Our results indeed support previously published reports and conclusions drawn from them (Lees et al., 2008; Wensky et al., 2005) and show that IFNγ-deficient mice predominantly succumb to the atypical form of EAE. However, we find IL17A to be dispensable for the occurrence of atypical EAE since animals deficient in both IFNγ and IL17A also display atypical disease symptoms (Figure 1a). To further investigate the atypical EAE course in DKO mice, we looked into the composition of immune cells infiltrating the CNS during the peak of disease. Based on previous reports, one would anticipate to find a large proportion of neutrophils in the CNS of mice with atypical EAE (Kroenke et al., 2010; Lees et al., 2008; Wensky et al., 2005). As expected, the CNS of IFNγ-deficient mice was heavily infiltrated by neutrophils and there was no difference found between the brain and the spinal cord (Figure 1b and 1c). Interestingly, the same is true for the DKO mice. This finding rules out one of the potential explanations of increased neutrophil count in the inflamed brain of mice with atypical EAE given by Stromnes et al., suggesting that the enhanced IL17A activity could explain the increased recruitment of neutrophils to the site. Apart from neutrophilia, we found additional characteristics of immune cell composition that correlated with the occurrence of atypical EAE - absence of

monocyte-derived dendritic cells (also termed moDCs), defined according to the surface phenotype of $CD45^+$ $CD11b^+$ $Ly6G^-$ $Ly6C^+$ $MHCII^+$ $CD11c^+$, and overall reduced proportion of infiltrating $CD11b^+$ $Ly6G^-$ cells.

That the neutrophils are actively recruited to the site of inflammation is most likely true nevertheless, since we could not observe any difference among the experimental groups in the amount of neutrophils circulating in the blood at day 7 after immunization, prior to disease onset (Figure 1d). Also, a simple assay of neutrophil activity and chemotactic responsiveness showed that neutrophils without the ability to sense IFNγ perform comparably to WT neutrophils in terms of responding to a potent immunogen (Figure 1e).

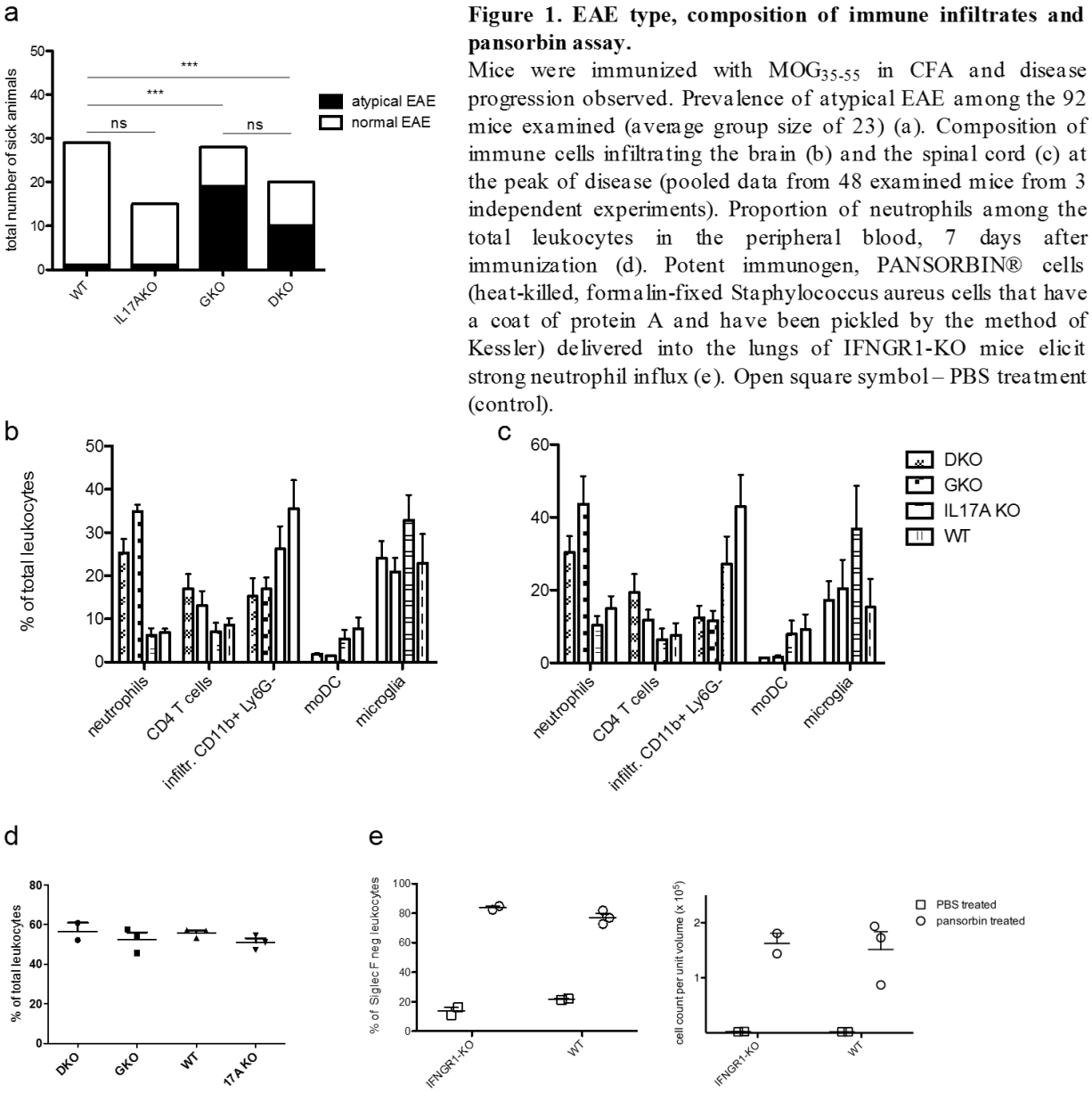

Figure 1. EAE type, composition of immune infiltrates and pansorbin assay.
Mice were immunized with MOG_{35-55} in CFA and disease progression observed. Prevalence of atypical EAE among the 92 mice examined (average group size of 23) (a). Composition of immune cells infiltrating the brain (b) and the spinal cord (c) at the peak of disease (pooled data from 48 examined mice from 3 independent experiments). Proportion of neutrophils among the total leukocytes in the peripheral blood, 7 days after immunization (d). Potent immunogen, PANSORBIN® cells (heat-killed, formalin-fixed Staphylococcus aureus cells that have a coat of protein A and have been pickled by the method of Kessler) delivered into the lungs of IFNGR1-KO mice elicit strong neutrophil influx (e). Open square symbol – PBS treatment (control).

It has been suggested that atypical EAE is dependent on GM-CSF secreted by CD4 T cells (Kroenke et al., 2010). Furthermore, it has been shown that all different "lineages" of encephalitogenic effector CD4 T cells predominantly secrete GM-CSF in the CNS, regardless of their signature cytokine before transfer (Codarri et al., 2011). We observed a similar effect in our experiments, where CD4 T cells in all 4 groups of mice predominantly secreted GM-CSF when examined at the peak of EAE disease (Figure 2). In fact, the only finding correlating to the occurrence of atypical EAE was absence of IFNγ, rather than any other change in the amount of cytokines commonly secreted by encephalitogenic CD4 T cells.

We have also examined the localization of immune cells infiltrating the CNS and the extent of tissue damage they inflict. As expected, histopathological analysis of IFNγ-deficient mice showed increased infiltration of immune cells into the cerebellum and meninges, with many granulocytes present. Microgliosis and demyelination are evident and more extensive than in the spinal cord. Almost identical observations were made in DKO mice, suggesting that DKO and GKO mice indeed have many features of EAE in common (Figure 3).

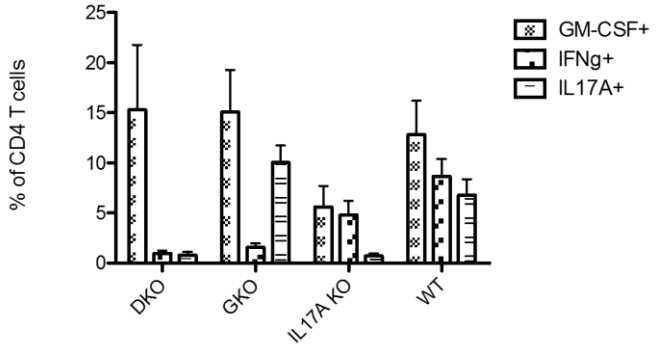

Figure 2. Cytokine production of CNS-infiltrating CD4 T cells.
Cells were isolated from the CNS of mice at the peak of EAE and processed according to the described procedure for intracellular cytokine staining. Pooled results from 2 independent experiments (31 mice).

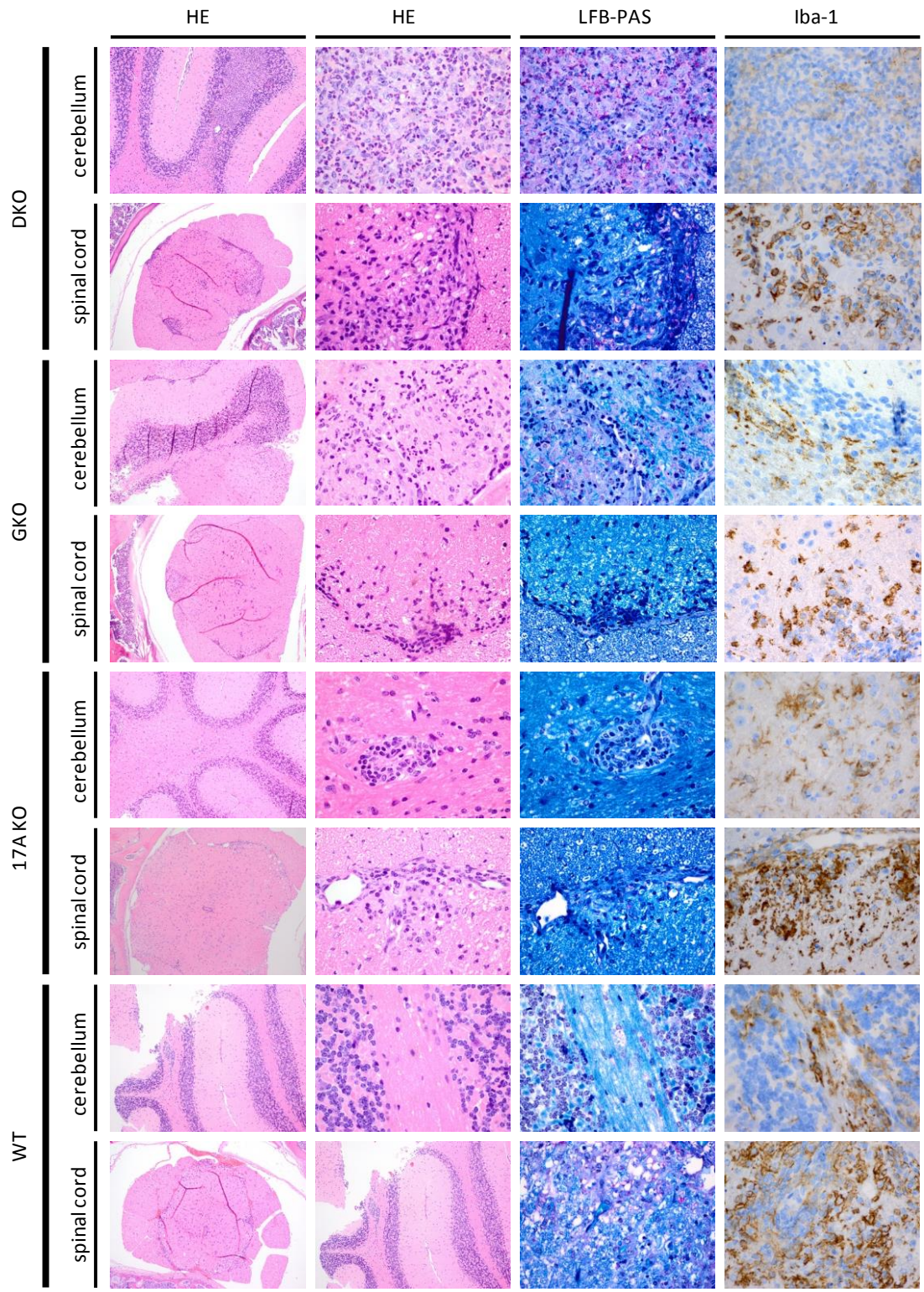

Figure 3. Histological analysis of the brain and spinal cord of DKO, GKO, IL17A KO and WT mice at the peak of EAE disease. Mice were sacrificed at the peak of disease and transcardially perfused with PBS, followed by formalin. CNS was harvested, embedded in paraffine and stained with hematoxylin and eosin, Luxol fast blue-periodic acid Schiff and Iba-1. First column 10x magnification. Columns 2-4 60x magnification. Representative pictures of 2 independent experiments are shown.

Atypical EAE seen in IFNγ deficient animals is a general phenotype of the absence of IFNγ-signaling during autoimmune inflammation

The finding that IL17A is dispensable for the occurrence of atypical EAE led us to hypothesize that the specific clinical features observed are not caused by the imbalanced cytokine secretion of effector CD4 T cells but rather by the inability of a particular cell type to sense IFNγ. To test the viability of this hypothesis, we have examined mice that carry no functional IFNγ receptor since they miss the alpha chain of the heterodimeric receptor complex (Huang et al., 1993). Upon active immunization, these mice predominantly develop atypical EAE and the composition of immune cells infiltrating the CNS is dominated by neutrophils, phenocopying the results from IFNγ-deficient mice (figure 4a and 4b). Taking into account also the work of Lees et al., who showed that there is no change in IL-17 production capacity regardless of the host's IFNγR expression level and clinical EAE manifestation (Lees et al., 2008), we conclude that the atypical EAE is a genuine phenotype of the absence of IFNγ signaling during autoimmune neuroinflammation and is not likely caused by the changes in relative amounts of cytokines normally secreted by CD4 T cells during neuroinflammation.

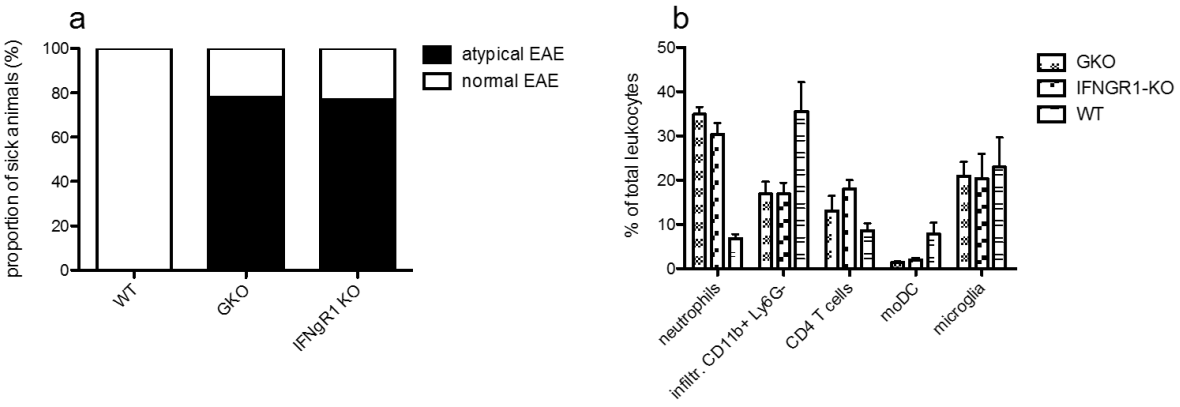

Figure 4. EAE type and the composition of immune infiltrates at the peak of disease
Mice were immunized with MOG_{35-55} in CFA and disease progression observed. Pooled data from 4 (a) and 2 (b) independent experiments is shown (41 and 28 mice, respectively). Prevalence of atypical EAE (a). Composition of immune cells infiltrating the CNS at the peak of EAE (b)

Phosphorylation of STAT1 can be used as a marker of IFNγ reactivity *in vivo*

Defining cell types able to respond to the IFNγ stimulus *in vivo* is compounded by the absence of a reliable antibody to detect surface presence of the IFNγ receptor beta chain and by the fact that many cell types are unresponsive to IFNγ in a steady state but acquire responsiveness during particular (patho)physiological conditions (Bernabei et al., 2001). We hypothesized that detection of STAT1 phosphorylation, an event downstream of the IFNγ receptor signaling, could potentially serve as a surrogate marker of IFNγ responsiveness. Knowing that STAT1 is not an intermediate in the IFNγ signaling cascade alone, we tested the hypothesis by actively inducing EAE in mice sufficient (WT) or deficient in IFNγ (GKO). The extensive imaging data

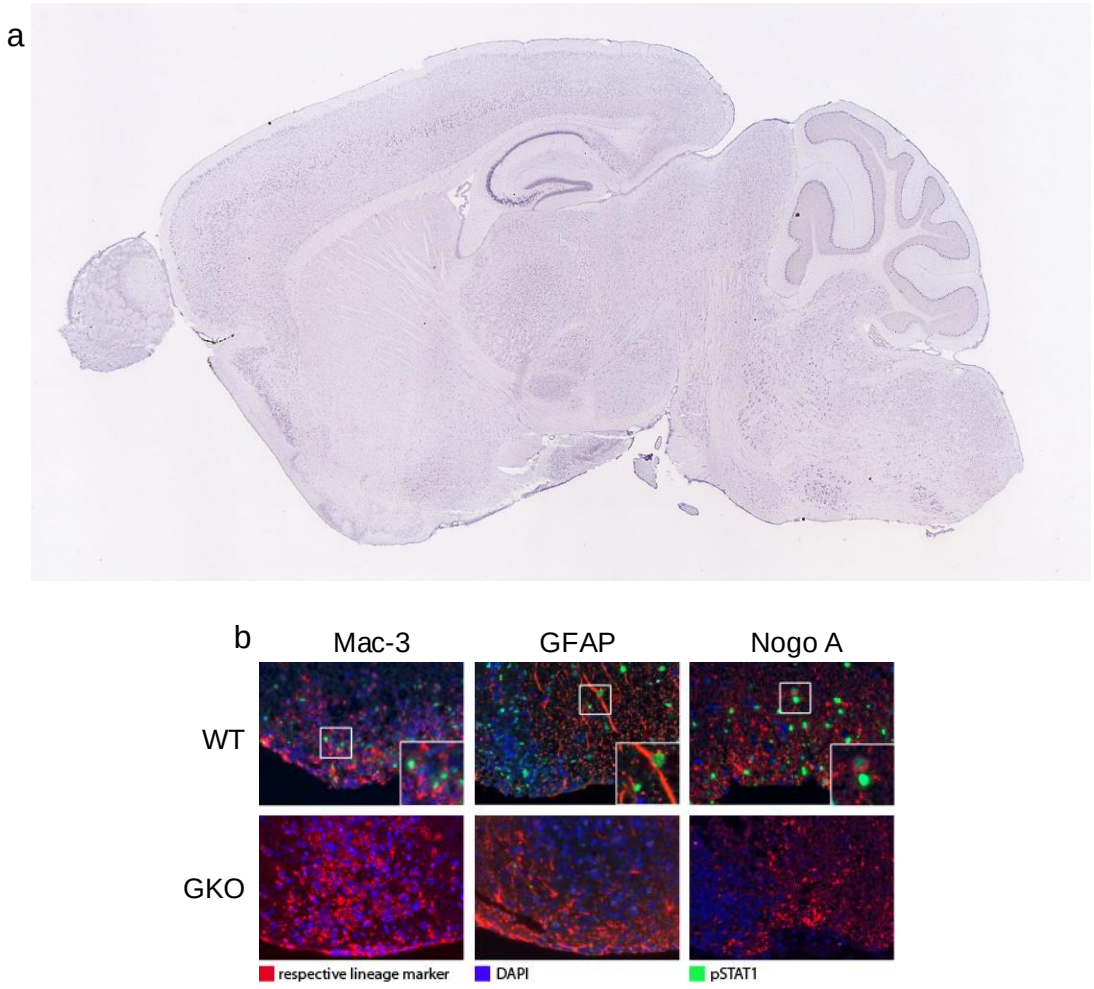

Figure 5. Abundance of ifngr2 mRNA in a naive mouse brain and pSTAT1 staining at the peak of EAE.
In situ hybridization of ifngr2 mRNA in mouse brain according to Allen Mouse Brain Atlas [Internet] ©2012 Allen Institute for Brain Science. Available from: http://mouse.brain-map.org/ (a). Immunohistochemistry of mouse brain at the peak of EAE (b) (13 mice examined).

available from the Allen Mouse Brain Atlas (Figure 5a) suggests that, in a resting mouse brain, the only cells capable of sensing IFNγ are found in the hippocampus and the layer of Purkinje cells in the cerebellum (Lein et al., 2007). However, during autoimmune inflammation of the CNS, all the cells in the brain (neurons, macroglia, microglia) stain positive for pSTAT1, suggesting potential responsiveness to IFNγ. This was confirmed in IFNγ-deficient mice, where pSTAT1 signal is completely absent (Figure 5b). This allows us to conclude that, at least during autoimmune inflammation of the CNS, no other cytokine is utilizing the STAT1 signaling pathway and detection of pSTAT1 could be used as a surrogate marker for IFNγ responsiveness *in vivo*. Extending our reasoning further, we showed that all the resident cells of the mouse brain could potentially be involved in driving the atypical EAE symptoms seen in IFNγ- or IFNγR-deficient mice.

Atypical EAE is largely mediated by a radio-resistant cell type

To better understand the requirements for IFNγ signaling in different cellular compartments, we created reciprocal bone marrow chimeras using wt and IFNγR1 KO mice and examined the form of EAE developed by each group. Lethally irradiated wild type mice were reconstituted with IFNγR1-deficient bone marrow and vice versa. In addition, as a control, lethally irradiated wild type and IFNγR1 mice were reconstituted with their respective syngeneic bone marrows (Figure 6). The efficiency of engraftment of donor's bone marrow was

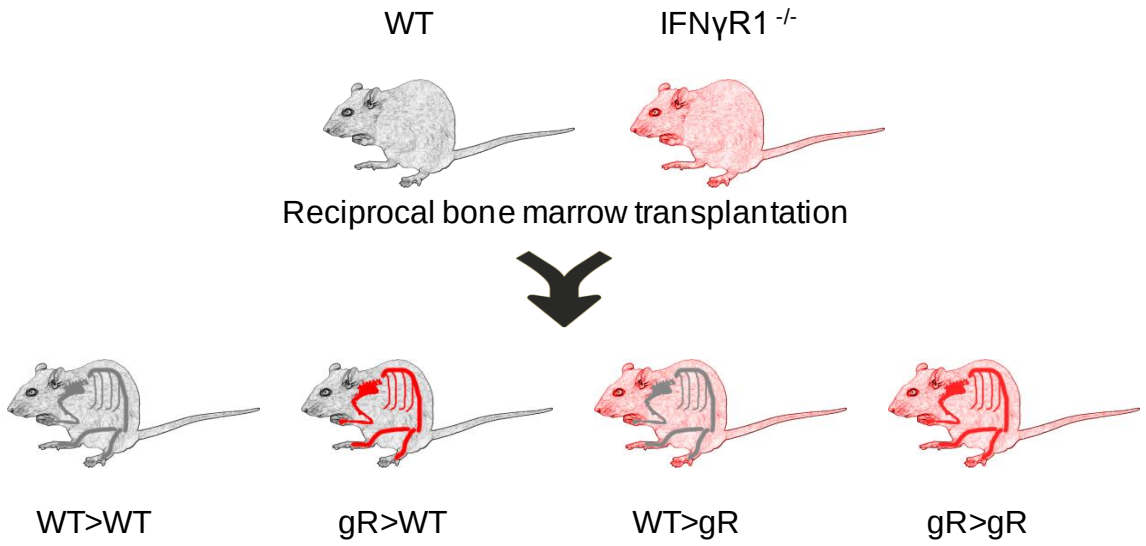

Figure 6. Schematic representation of the bone marrow chimera experiment
Recipient mice were lethally irradiated with 1100 rads (split dose) and intravenously injected with 10 × 10^6 donor BM cells. Engraftment took place over the following 6 weeks of recovery

found to be 94% on average as assessed by flow cytometric analysis of congeneic markers expressed on the surface of peripheral blood leukocytes (data not shown).

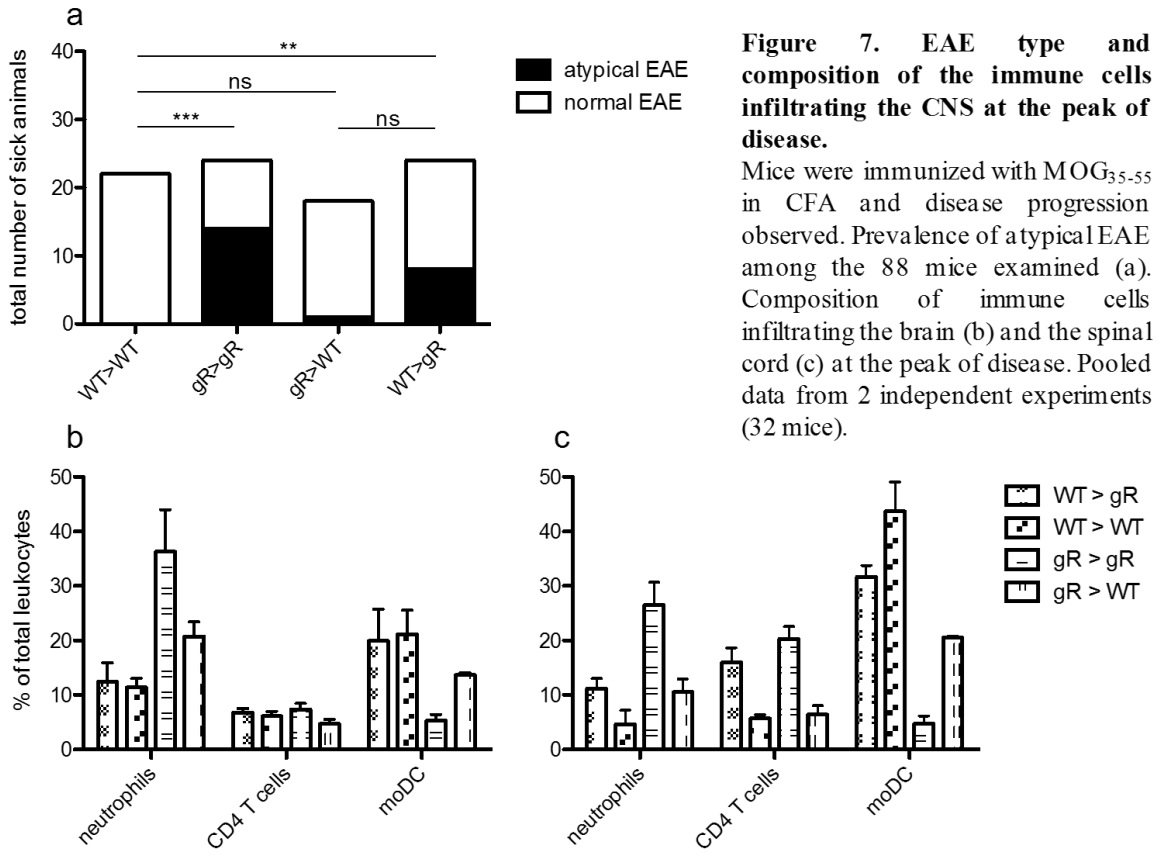

Figure 7. EAE type and composition of the immune cells infiltrating the CNS at the peak of disease.
Mice were immunized with MOG_{35-55} in CFA and disease progression observed. Prevalence of atypical EAE among the 88 mice examined (a). Composition of immune cells infiltrating the brain (b) and the spinal cord (c) at the peak of disease. Pooled data from 2 independent experiments (32 mice).

Upon active EAE induction we observed higher prevalence of atypical EAE in the IFNγ receptor deficient mice reconstituted with WT bone marrow than vice versa (Figure 7a). Comparison to the control groups led us to conclude that the deficiency of IFNγ signaling in the radiation-resistant cell compartment contributes more to the occurrence of atypical symptoms than deficiency in the hematopoietic compartment.

The flow cytometric analysis revealed very high granulocyte count in the brains and spinal cords of the gR>gR group, with other groups showing moderate amounts. The moDCs were also found at lowest proportion in the brain and spinal cord of the gR>gR group, with other groups showing higher numbers.

Anticipated result would have been a clear accordance of findings in WT>WT and gR>gR (control groups) with the ones reported previously in WT and GKO mice, respectively, and each experimental group fitting to one of the

control groups. While the composition of immune cell infiltrates in WT>WT and gR>gR in terms of neutrophils and moDCs indeed resembled the ones found in WT and GKO mice, both experimental groups resembled more the WT>WT control group. This was in contrast to the EAE features, where, looking at the prevalence of atypical EAE in the experimental groups, WT>gR resembled more the gR>gR and gR>WT resembled more the WT>WT group, suggesting a bigger role for IFNγ signaling in the radiation-resistant compartment.

Histopathological analysis of the CNS revealed extensive immune cell infiltrates, microgliosis and demyelination in all experimental groups. Substantial presence of granulocytes was observed in all groups apart from WT>WT. The analysis did not reveal any particular difference in the intensity of inflammation between the spinal cord and the brain of each group. Also, the analysis did not support a notion that some groups showed higher extent of inflammation than the others - the specimens with highest scores for intensity of inflammation were found in all groups (Figure 8).

Overall, the results suggest that the deficiency of IFNγ signaling in the radiation-resistant cell compartment contributes more to the occurrence of atypical symptoms than the deficiency in the hematopoietic compartment. The fact that the composition of immune cells infiltrating the CNS does not fully conform to this line of reasoning raises the question of whether there is a causal link between these two sets of features or to they just happen to correlate most of the time for reasons not yet understood. Some authors suggest there is no causal link between the two (Kroenke et al., 2010; Lee et al., 2012).

Following the same line of reasoning as Kroenke et al. and Lee at al., we decided that greater weight should be assigned to correlated clinical features, rather than the composition of the cells infiltrating the CNS since atypical clinical course has been shown to reflect differential localization of dominant site of CNS tissue damage. As we saw similar clinical features in the WT>gR and in the gR>gR control group, we concluded that the disruption of the IFNγ signaling in a certain radiation-resistant cell type might be responsible for development of atypical EAE.

In order to investigate which cell type is is involved in the process, we decided to use a genetic system for cell-specific ablation of IFNγ signaling by targeting Cre-mediated disruption of the *fingr2* gene to different radiation-resistant cell types that might play a role during EAE.

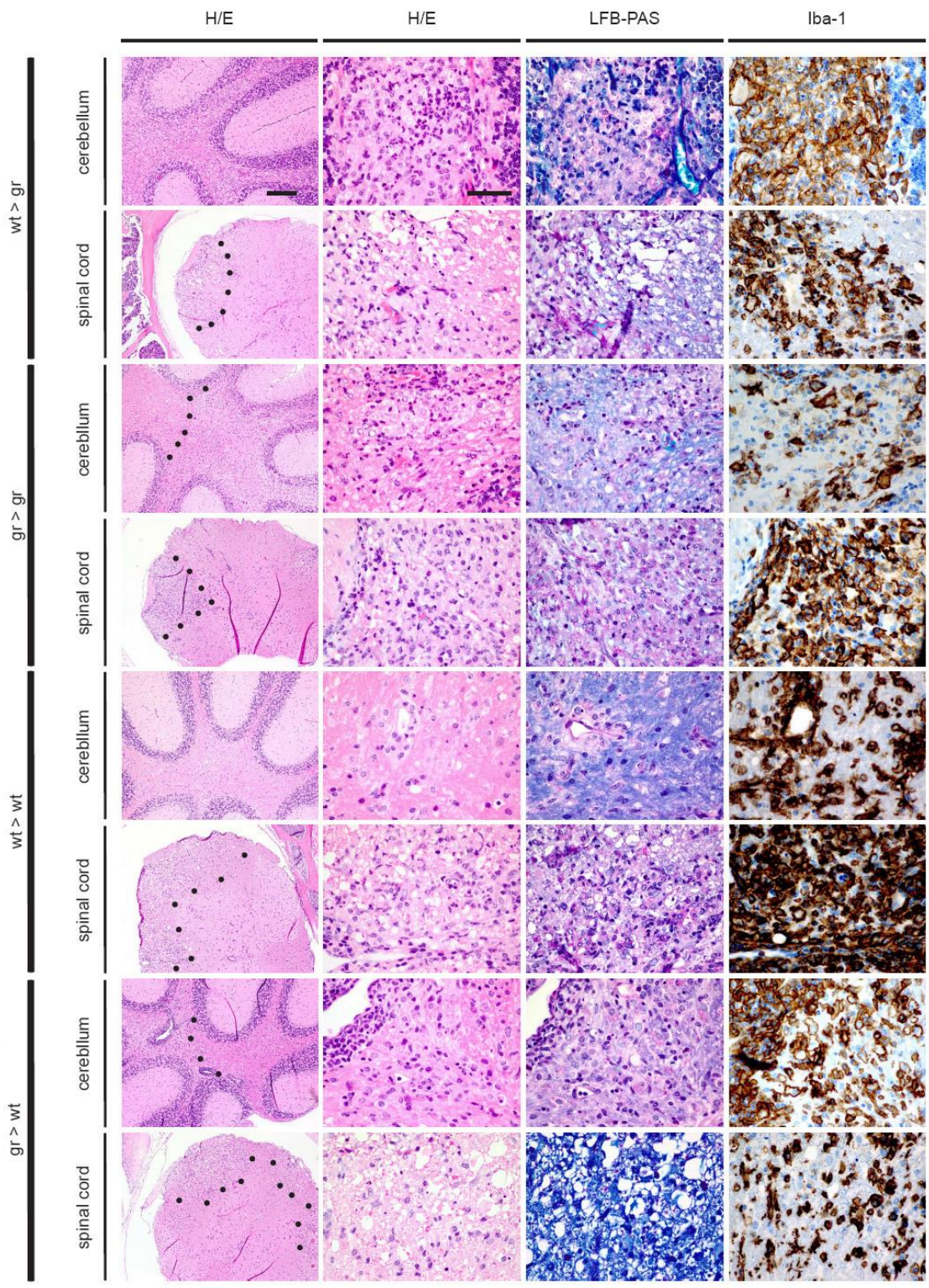

Figure 8. Histological analysis of the brain and spinal cord of bone marrow chimeric mice at the peak of EAE.

Mice were sacrificed at the peak of disease and transcardially perfused with PBS, followed by formalin. CNS was harvested, embedded in paraffine and stained with hematoxylin and eosin, Luxol fast blue-periodic acid Schiff and Iba-1. First column 10x magnification. Columns 2-4 60x magnification. Representative pictures of 2 independent experiments are shown (32 mice).

The conditional IFNgR2 genetic system is a valid tool to study cell-specific ablation of IFNγ signaling

The group of Werner Muller from the University of Manchester has generated a mouse strain where the exon 4 of the ifngr2 gene is flanked by loxP sites (*Ifngr2*^{tm1Hzi}, unpublished). Breeding this mouse strain to strains with cell-specific expression of the Cre recombinase will allow the abrogation of IFNγ signaling in that particular cell type(s).

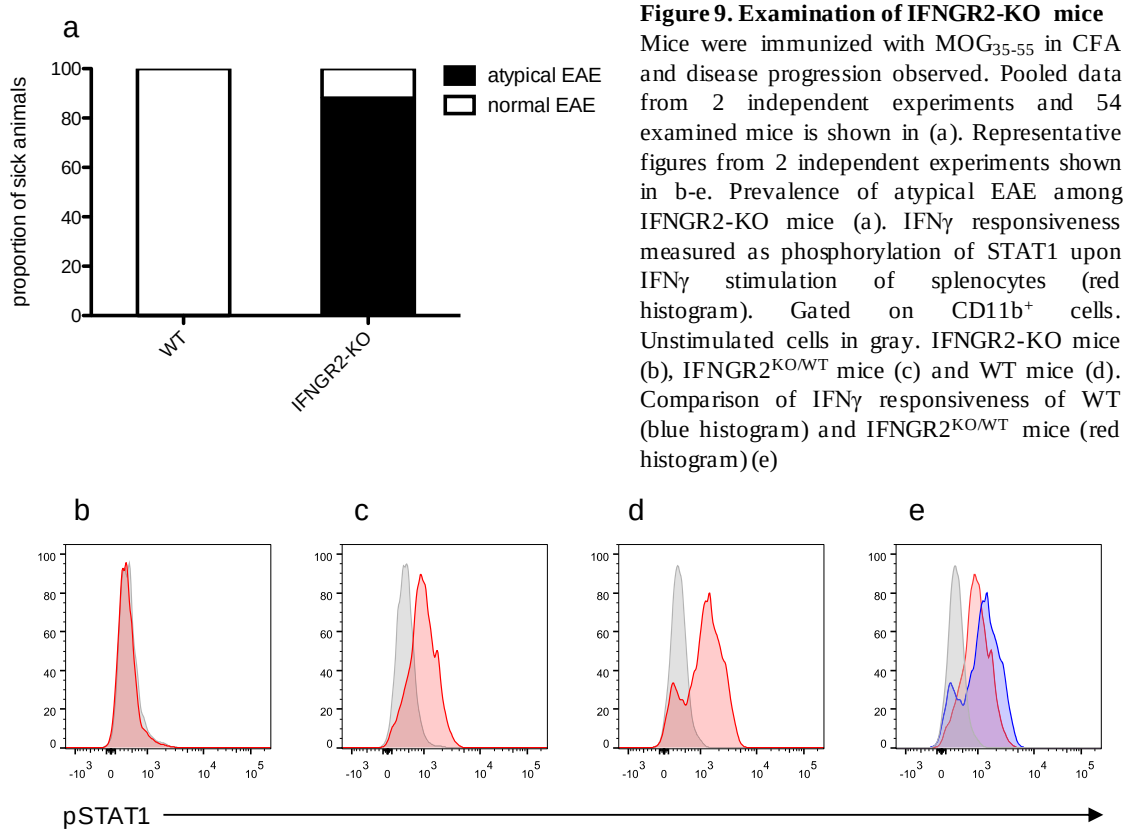

Figure 9. Examination of IFNGR2-KO mice
Mice were immunized with MOG_{35-55} in CFA and disease progression observed. Pooled data from 2 independent experiments and 54 examined mice is shown in (a). Representative figures from 2 independent experiments shown in b-e. Prevalence of atypical EAE among IFNGR2-KO mice (a). IFNγ responsiveness measured as phosphorylation of STAT1 upon IFNγ stimulation of splenocytes (red histogram). Gated on $CD11b^+$ cells. Unstimulated cells in gray. IFNGR2-KO mice (b), $IFNGR2^{KO/WT}$ mice (c) and WT mice (d). Comparison of IFNγ responsiveness of WT (blue histogram) and $IFNGR2^{KO/WT}$ mice (red histogram) (e)

To test if the mentioned strain indeed behaves as expected, we crossed it to a mouse strain that ubiquitously expresses the Cre-recombinase, namely deleter-cre (Schwenk et al., 1995), effectively producing a complete IFNγ-receptor knock out, subsequently termed IFNGR2-KO mice.

Upon active EAE induction, this newly generated mouse strain showed atypical disease symptoms, completely phenocopying the clinical features of the IFNγ- and IFNγ receptor-deficient mice (Figure 9a). Based on just a few animals examined, an increase of neutrophils among the immune cells infiltrating the CNS compared to wt mice was not observed (data not shown). It should also be noted that these mice seemed to have a slightly milder course of atypical EAE,

never reaching scores of 4 or 5, as it was sporadically the case with IFNGR1-KO mice. Additionally, as the mice were sacrificed at the late stage of the disease (25 days after immunization), unlike the GKO and IFNGR1 KO mice, taken earlier, this could be a potential explanation for the absence of neutrophilia in the inflamed CNS (Abromson-Leeman et al., 2004). Whether this really was the case remains to be established in subsequent experiments investigating infiltrates at different time points in GKO, IFNGR1-KO and IFNGR2-KO mice.

Stimulation of splenic myeloid cells, previously shown to express functional IFNγ receptor at high levels in a steady state in humans (Bernabei et al., 2001) and mice (Heng et al., 2008), with recombinant mouse IFNγ revealed complete absence of IFNγ signaling in IFNGR2-KO mice, confirming the genetic makeup of the newly created strain (Figure 9b). The heterozygous animals, still having one non-recombined (wt) *ifngr2* locus showed a uniform pSTAT1 staining, with just slightly lower overall staining intensity compared to the WT mice (Figure 9c and 9e). For practical purposes, it suggests that the heterozygous mice show equal response to IFNγ stimulation as wt mice. Overall, these results suggest that the ifngr2 flox genetic system is functional and represents an appropriate tool for further studying the cell-specific deletion of the *ifngr2* gene.

Disruption of IFNγ signaling in neurons, astrocytes and oligodendrocytes is insufficient to drive atypical EAE

Some previous reports suggested, based on adoptive transfer experiments where WT cells were transferred into IFNγ-receptor-deficient hosts, that the atypical EAE is very likely mediated by some cell type(s) other than the adoptively transferred CD4 T cells (Lees et al., 2008). Even older reports imply that inability of a recipient mouse to sense IFNγ causes a more severe form of EAE (Willenborg et al., 1996). At the same time, our own results obtained from BM chimeras suggested that the absence of IFNγ signaling in the radiation-resistant cell compartment is likely contributing more to the atypical phenotype than the same defect in hematopoietic cells. Also, immunohistological examination of the inflamed CNS in WT mice showed that almost all cell types of the CNS are responsive to IFNγ during autoimmune inflammation. This led us to hypothesize that the inability of the invaded tissue (CNS) to respond to the IFNγ mainly secreted by the invading CD4 T cells could be a initiating event for atypical EAE. We have decided to test the hypothesis by selectively disrupting the *ifngr2* gene in one or more cell types of the CNS.

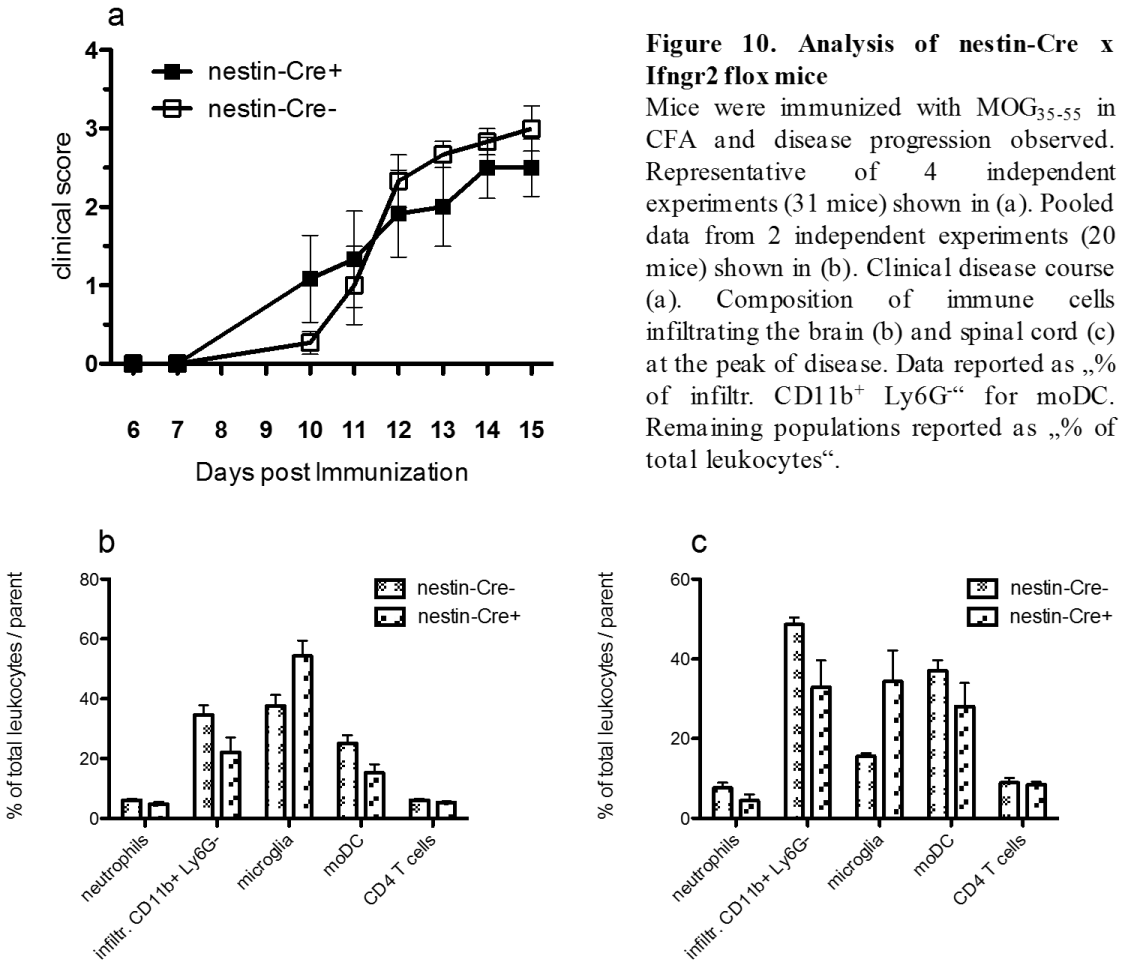

Figure 10. Analysis of nestin-Cre x Ifngr2 flox mice
Mice were immunized with MOG_{35-55} in CFA and disease progression observed. Representative of 4 independent experiments (31 mice) shown in (a). Pooled data from 2 independent experiments (20 mice) shown in (b). Clinical disease course (a). Composition of immune cells infiltrating the brain (b) and spinal cord (c) at the peak of disease. Data reported as „% of infiltr. $CD11b^+$ Ly6G" for moDC. Remaining populations reported as „% of total leukocytes".

The nestin-Cre mouse line (Tg(Nes-cre)1Kag) carries the Cre recombinase gene under the control of the nestin promoter and enhancer, which specifically directs expression in neural precursor cells (Isaka et al., 1999; Zimmerman et al., 1994). In situ hybridization analysis demonstrated that the Cre recombinase is specifically expressed in the developing nervous system. The expression was clearly observed in the peripheral nervous system such as the dorsal root ganglia and in the ventricular zone of the developing central nervous system such as the telencephalon and the spinal cord (Isaka et al., 1999).

We bred the nestin-Cre strain to *Ifngr2*^{tm1Hzi} mice, generating a new strain that lacks a functional IFNγ receptor on the surface of brain cells descending from neural precursor cells - neurons and glia cells (apart from microglia, which originates from primitive, yolk sac macrophages (Geissmann et al., 2010; Schulz et al., 2012; Yona et al., 2013)). The efficiency and specificity of Cre-mediated recombination at the *Ifngr2* locus in neurons and/or glia cells was not confirmed (see Discussion for more details).

A total of 6 independent EAE experiments (active EAE induction) were performed, with somewhat inconsistent results in terms of the occurrence of atypical EAE. In 4 out of 6 experiments, active EAE induction was followed with classical disease symptoms of comparable magnitude in both experimental groups, suggesting that the deficiency of IFNγ signaling in neurons and macroglia (a collective term used for astrocytes and oligodendrocytes) is not sufficient to drive atypical disease (Figure 10a). The notion was further backed up with the analysis of the composition of immune cell infiltrates in the CNS of diseased animals. We found no significant difference among the experimental groups and none of them displayed an increased proportion of neutrophils or absence of moDCs, features usually observed during atypical EAE in IFNγ- and IFNγR-deficient mice (Figure 10b and 10c).

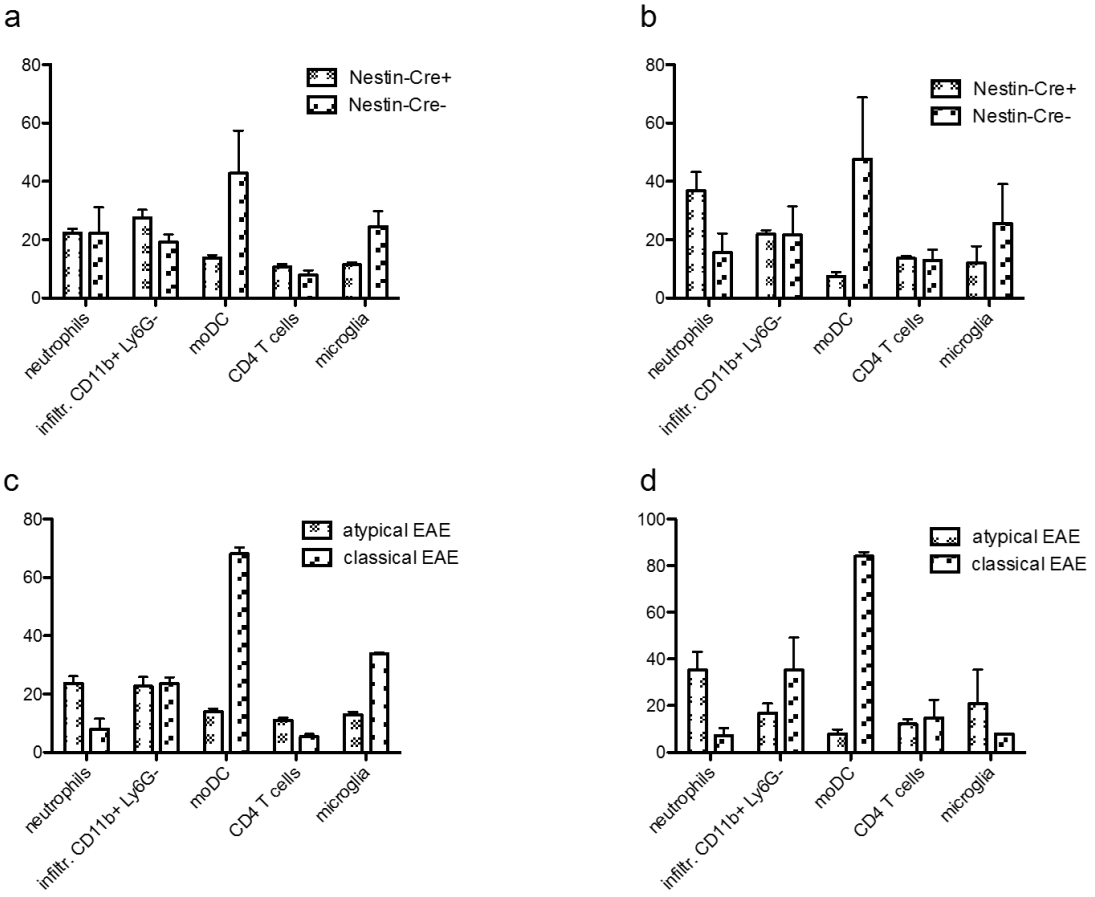

Figure 11. Analysis of 2 aberrant experiments with nestin-Cre x Ifngr2 flox mice
Mice were immunized with MOG_{35-55} in CFA and leukocytes infiltrating the CNS isolated at the peak of disease. Composition of immune cells infiltrating the brain (a) and the spinal cord (b) of mice from 2 abberant EAE experiments (12 mice) where signs of atypical EAE were observed. Data is grouped according to the animal genotype (absence/presence of Cre-recombinase).
The same data is shown in a lower row, with brain in (c) and spinal cord in (d), but grouped according to the observed EAE type.
Data reported as „% of infiltr. $CD11b^+$ Ly6G-" for moDC. Remaining populations reported as „% of total leukocytes".

The remaining two independent experiments showed, however, the occurrence of atypical EAE (data not shown) that did not correlate with the genotype. Fischer's exact test reported no statistical difference between the Cre^+ and Cre^- mice (data not shown). The analysis of immune cell infiltrates showed resemblance to the composition normally found during prototypical atypical EAE seen in IFNγ- or IFNγ receptor-deficient mice (low proportion of monocyte-derived DCs and elevated proportion of neutrophils, Figure 11a and 11b). A k-means clustering analysis defined two clusters of variables, where genotype was not the main grouping variable but rather the feature of EAE. That means increased numbers of neutrophils were found in mice with atypical EAE irrespective of the genotype. When grouping the results obtained from analysis of composition of cells infiltrating the CNS according to this variable (Figure 11c and 11d), the overall variation within the groups was minimized, suggesting a stronger correlation of immune cell composition to the disease phenotype, not the animal genotype. Based on our data, previous published reports and our unpublished observations, we have a reason to suspect a germline deletion of the *Ifngr2* gene in these mice which will be discussed in more detail later in the Discussion.

Overall, we concluded that disruption of IFNγ signaling on neurons and macroglia is insufficient to drive atypical EAE.

Cell-specific disruption of IFNγ signaling in microglial cells is insufficient to drive atypical EAE

The nestin-Cre strain directs Cre-recombinase activity in almost the entire brain apart from microglia cells. Our previous results suggest that microglia cells are responsive to IFNγ during neuroinflammation, making them a cell type with a potential role in atypical EAE. To investigate the effects of disrupted IFNγ signaling in microglia on EAE disease course, we have used the CX_3CR1^{CreER} mouse strain. It harbors the targeted insertion of a conditional active Cre-ERT2 recombinase into the Cx3cr1 locus, replacing the coding exon of Cx3cr1. Upon administration of tamoxifen more than 95% of both brain and spinal cord microglia were shown to express the reporter gene construct. Thus, when crossed to mice harboring "floxed" candidate genes, the system can be utilized to delete or express specific genes in CX_3CR1^+ microglia (Yona et al., 2013). Certain lymphocyte and myeloid subsets also express CX_3CR1, making them a target of CreER-mediated genetic rearrangement. However, most peripheral myeloid cells

that undergo Cre activation and rearrangements have a limited half-life and are continuously replaced by BM-derived cells. Genetic modifications are thus progressively lost with time in these populations. In contrast, the resident microglia pool which self-renews without further input from the BM retains once introduced gene modifications throughout the life of the organism (Wolf et al., 2013).

We bred the CX_3CR1^{CreER} mice to the *Ifngr2*^{tm1Hzi} mice, generating a new strain that, upon tamoxifen treatment, undergoes Cre-mediated recombination and renders microglia and a subset of circulating leukocytes unresponsive to IFNγ stimulus. After allowing for the repopulation of targeted circulatory cells from their respective BM precursors, the mouse strain remains with only microglia not having a functional IFNγ receptor anymore. The efficiency of Cre-mediated disruption of the "floxed" locus in microglial cells was confirmed using the genetic reporter system (EYFP expression following Cre-mediated recombination event) and was found to be around 71% (average of 5 different mice, Figure 12c).

Active EAE induction in the newly created strain results in the typical disease progression without observing any enduring atypical symptoms that correlated with the genotype. The disease course and overall severity do not differ among the two experimental groups (CX_3CR1^{CreER+} and CX_3CR1^{CreER-}). Additionally, the cellular composition of the immune infiltrates in the CNS at the peak of EAE does not differ among the two experimental groups and resembles the one found in WT mice rather than the one found in complete IFNγ receptor-deficient mice. Taken together, the results indicate that the disruption of IFNγ signaling in microglial cells alone is insufficient to drive atypical disease course as seen in complete IFNγ receptor-deficient or IFNγ-deficient mice.

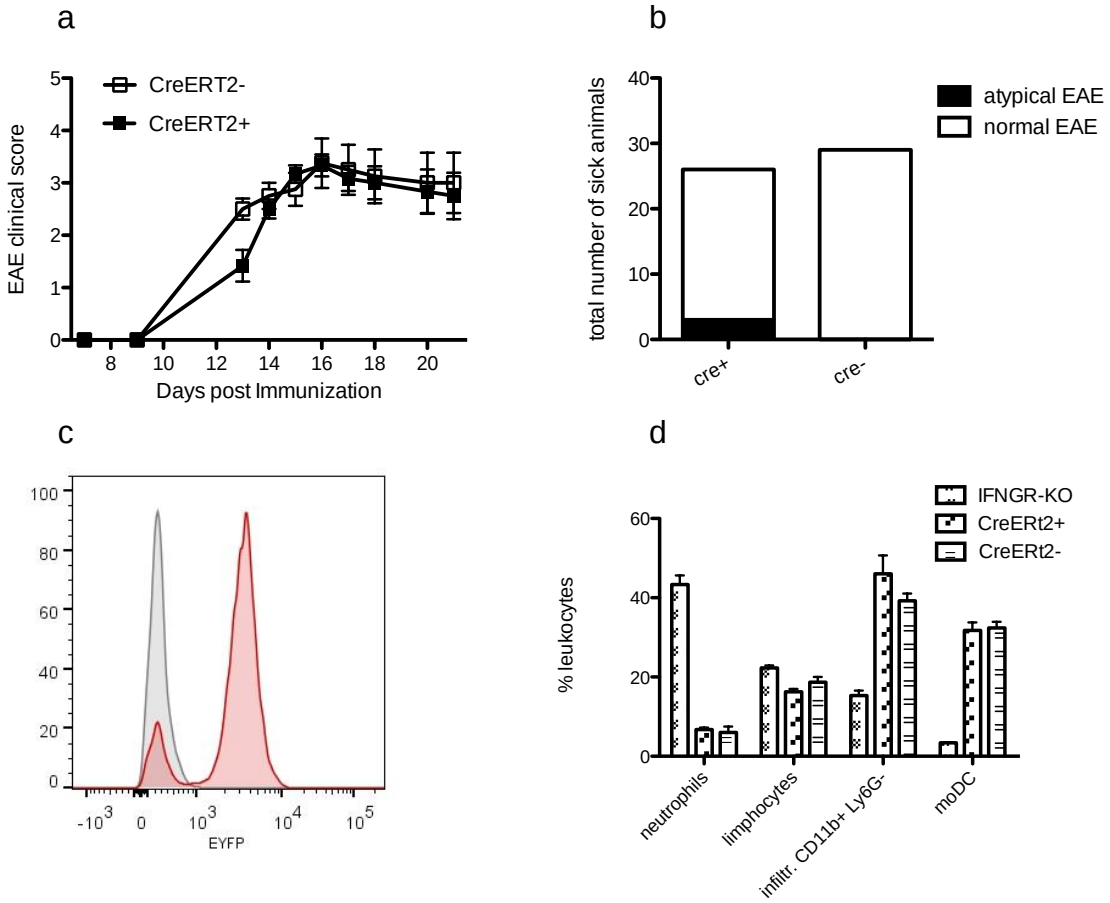

Figure 12. Analysis of CX3CR1-CreERt2 x Ifngr2 flox mice
Cre expression was induced according to the Methods section. Mice were immunized with MOG_{35-55} in CFA and disease progression observed. Representative of 5 independent experiments (44 mice) shown in (a). Pooled data from 2 independent experiments (30 mice) shown in (d). Representative figure (5 mice) shown in (c).
Clinical disease course (a). Prevalence of atypcial EAE (b) Efficiency of CreERt2 expression in microglia following Tamoxifen induction, measured as percentage of cells expressing the reporter gene construct (flox-stop-flox EYFP). In red are $CreERT2^+$ and in gray $CreERT2^-$ microglia (c). Composition of immune cells infiltrating the CNS at the peak of disease (d).

Cell-specific disruption of IFNγ signaling in endothelial cells is insufficient to drive atypical EAE

The disruption of the blood-brain barrier (BBB) has been shown to precede the onset of clinical symptoms in EAE and the sites of disruption correlate with the sites of inflammatory cell accumulation (Bennett et al., 2010). Since endothelial cells make a critical component of BBB and they have been also shown to respond to IFNγ stimulation (Schroder et al., 2004), we sought to investigate whether disruption of IFNγ signaling in endothelial cells plays a role in driving atypical EAE. To this end, we have used the VE-Cadherin-Cre mouse strain, which harbors a transgene that contains the Cre recombinase coding

region fused to a regulatory region of the VE-Cadherin promoter (Alva et al., 2006). Vascular endothelial cadherin (VECadherin), also known as CD144 and cadherin-5, is a transmembrane protein involved in endothelial homotypic cell adhesion (Lampugnani et al., 1995). During initial characterization, Cre expression was reported to increase throughout embryonic development, starting as early as E7.5 and reaching close to full penetrance (96.4%) by E14.5. It was equally penetrant in arteries, veins and capillaries and was specific for endothelial cells. The ectopic expression of the reporter construct was not found, nor expression of the reporter in Cre-negative mice or variations in the pattern of Cre activity when the lines were crossed into C57Bl/6 mice for 8 generations(Alva et al., 2006). In adult mice, reporter expression was also found in about half of all circulating blood cells and in hematopoietic organs, with approximately 50% of all hematopoietic lineages in adult bone marrow expressing the reporter (Alva et al., 2006).

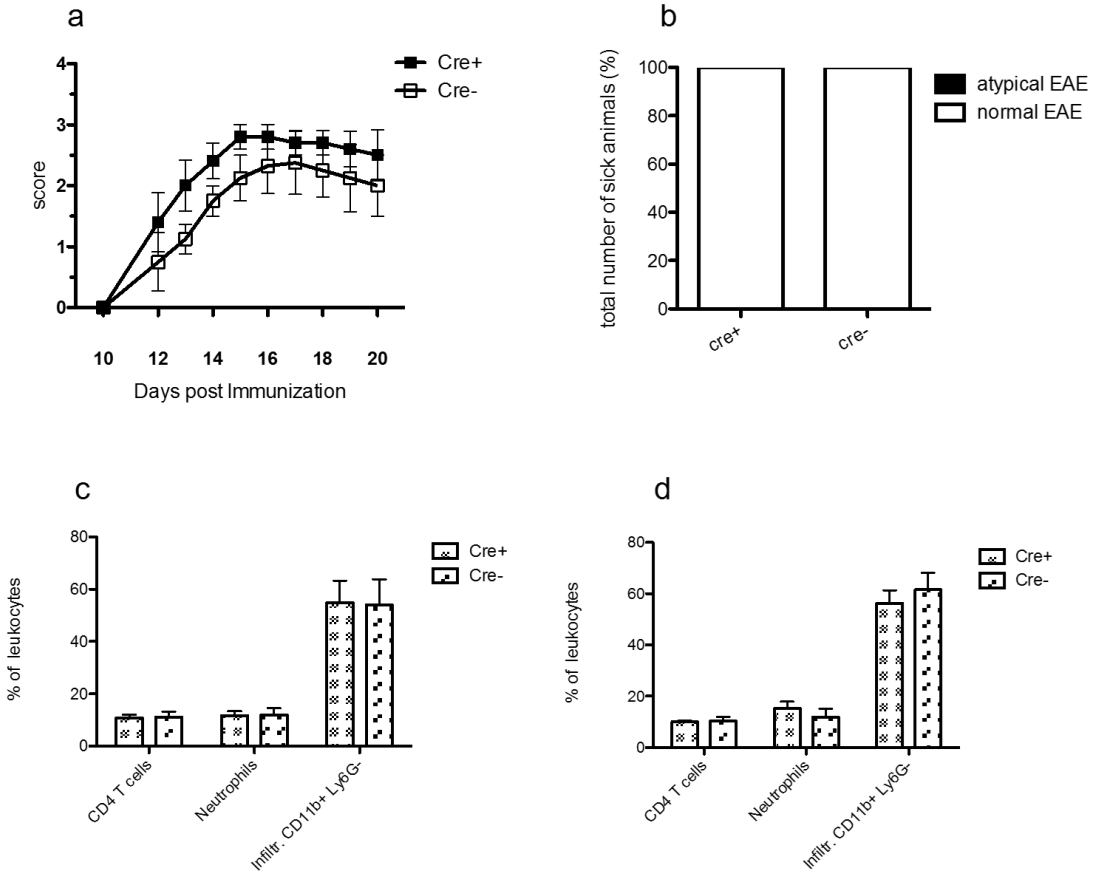

Figure 13. Analysis of VE-Cadherin-Cre x Ifngr2 flox mice
Mice were immunized with MOG_{35-55} in CFA and disease progression observed. Representative of 4 independent experiments (32 mice) shown in (a). Pooled data from 2 independent experiments (20 mice) shown in (c) and (d).
Clinical disease course (a). Prevalence of atypcial EAE (b) Composition of the immune cells infiltrating the brain (c) and the spinal cord (d) at the peak of disease.

We bred the VE-Cadherin-Cre strain to *Ifngr2*^{tm1Hzi} mice, generating a new strain that lacks a functional IFNγ receptor on the surface of endothelial cells and a subset of blood-circulating leukocytes. The efficiency of Cre-mediated recombination at the *Ifngr2* locus in endothelial cells was confirmed using the *in vivo* assay of ICAM-1 upregulation in a tumor microenvironment and was shown to be complete (Sara Burkhard, unpublished). Active EAE induction in this newly created strain results in the classic disease progression without observing any atypical symptoms. The disease course and overall severity do not differ among the two experimental groups (Tg$^{VE\text{-}Cadherin\text{-}Cre+}$ and Tg$^{VE\text{-}Cadherin\text{-}Cre-}$). The cellular composition of the immune infiltrates in the CNS at the peak of disease does not differ among the two experimental groups and resembles the one found in WT mice rather than the one found in complete IFNγ receptor-deficient mice (Figure 13). Additionally, both experimental groups show no difference in the composition of immune cell infiltrates in the brain and brain stem compared to the spinal cord and both sites show the same ratio of infiltrating cells to microglia, suggesting the same extent of immune cell invasion of both sites (data not shown). Taken together, the results indicate that the ablation of IFNγ receptor from the surface of endothelial cells alone is insufficient to drive atypical disease course as seen in a complete IFNγ receptor-deficient or IFNγ-deficient mice.

Cell-specific disruption of IFNγ signaling in T cells is insufficient to drive atypical EAE

Since we were able to show that disruption of IFNγ signaling in almost all the radiation-resistant cells of the CNS is insufficient to drive atypical EAE on their own, we decided to investigate a few additional cell compartments. Namely, some cell types of the immune system seem to be somewhat resistant to the irradiation regime we used previously in generating bone marrow chimeric mice. It has been shown by others (Iva Lelios and Melanie Greter, personal communication) that, in some cases, the CD4 T cell compartment shows only a 50% reconstitution efficiency following lethal irradiation and transfer of congeneic bone marrow, despite the fact that other cell compartments showed a 100% reconstitution efficiency. To investigate whether disruption of IFNγ signaling in T cells plays a role in driving atypical EAE, we used the CD4-Cre mouse strain that harbors a transgene that contains the *CD4* enhancer, promoter and silencer sequence driving the expression of the *Cre* recombinase gene. Cre

expression reportedly results in >99% deletion of *loxP* flanked genes by the double-positive stage of T-cell development (Lee et al., 2001).

We bred CD4-Cre mice to *Ifngr2*^{tm1Hzi} mice, thus generating a new strain that lacks a functional IFNγ receptor on the surface of T cells. The efficiency of Cre-mediated recombination at the *Ifngr2* locus was confirmed using the *in vitro* assay of IFNγ responsiveness of splenic CD4 T cells and was shown to be >99% (Figure 14c).

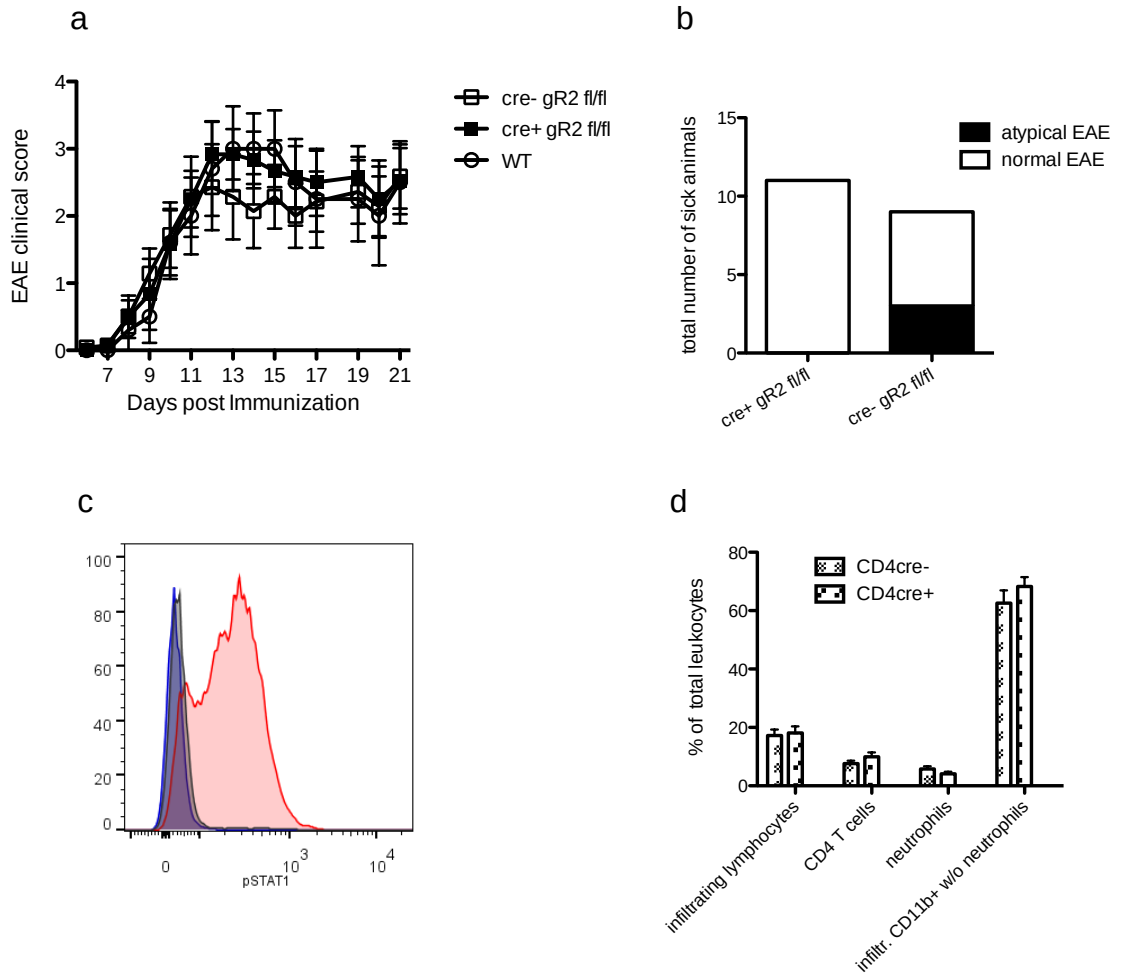

Figure 14. Analysis of CD4-Cre x Ifngr2 flox mice
Mice were immunized with MOG_{35-55} in CFA and disease progression observed. Representative of 3 independent experiments (30 mice) shown in (a). Pooled data from 2 independent experiments (20 mice) shown in (d).
Clinical disease course (a). Prevalence of atypical EAE in CD4-Cre$^+$ and CD4-Cre$^-$ mice (b). Efficiency of Cre-mediated disruption of Ifngr2 gene directly estimated through *ex vivo* stimulation of splenic T cells with IFNγ (in red), followed by detection of phosphorylated STAT1 (c). Unstimulated splenic T cells in gray. Stimulated IFNGR1-KO splenic T cells in blue. Composition of the immune cells infiltrating the CNS at the peak of disease (d).

Active EAE induction in the newly created strain results in the typical disease progression without observing any enduring atypical symptoms that

correlated to the animal genotype (Figure 14b). The disease course and overall severity do not differ among the two experimental groups (Tg(CD4-Cre)+ and Tg(CD4-Cre)-) and resemble the ones of the WT mouse strain (Figure 14a). Additionally, the cellular composition of the immune infiltrates in the CNS at the peak of disease does not differ among the two experimental groups and resembles the one found in WT mice rather than the one found in complete IFNγ receptor-deficient mice (Figure 14d). Taken together, the results indicate that the ablation of IFNγ receptor from the surface of T cells alone is insufficient to drive atypical disease course as seen in a complete IFNγ receptor-deficient or IFNγ-deficient mice.

Cell-specific disruption of IFNγ signaling in neutrophils and a subset of myeloid cells drives atypical EAE

Since all attempts made to this point have failed to induce atypical EAE by disrupting IFNγ signaling in various cell types that are important during EAE and also have been shown to respond to IFNγ *in vivo* during neuroinflammation, we have decided to target the disruption of IFNγ signaling to the two cell types that have so far shown differential abundance among the cells infiltrating the CNS during classical and atypical EAE - neutrophils and moDC. Probably the most appropriate tool for this is the LysM-Cre mouse strain. It has a targeting construct encoding for the Cre-recombinase introduced into the endogenous ATG-start site within the first exon of the lysozyme M gene, driving its specific expression in monocyte/macrophages and neutrophils (Clausen et al., 1999).

During initial characterization, the Cre expression was reported in 95% of $F4/80^+$ peritoneal macrophages and 99% of peritoneal neutrophils. Lymph node T cells and splenic B cells were shown not to undergo significant Cre-mediated gene rearrangement (2%). Around 16% of splenic DCs showed evidence of Cre activity (Clausen et al., 1999). Subsequently, activity of LysM-Cre was also shown in hematopoietic stem cells, allowing for Cre-mediated genetic rearrangement being detected in myelomonocytic cells and subset of B and T cells. In extreme cases, the entire hematopoietic compartment exhibited Cre-mediated reporter gene expression (Ye et al., 2003). A different group of authors reported LysM-Cre-mediated reporter gene (EYFP) expression in 55% of Ly-$6C^{hi}$ monocytes and 75% of Ly-$6C^{lo}$ monocytes. However, they suggest that there is no inherent difference among the $EYFP^-$ and $EYFP^+$ monocytes. The difference is rather

caused by the stochastic failure of lysozyme promoter or Cre-mediated excision (Jakubzick et al., 2008).

We bred the LysM-Cre strain to *Ifngr2*^{tm1Hzi} mice, generating a new mouse strain with disrupted IFNγ signaling in neutrophils and a subset of myelomonocytic cells.

Active EAE induction in this newly created strain results in consistent occurrence of atypical EAE with average prevalence of 48% (10 out of 21 mice in total, Figure 15a). The control group has a prevalence of 8% (2 out of 26 mice in total). According to the Fischer's exact test, this difference is statistically significant with a P value of 0.0026. The course of atypical EAE in LysM-Cre$^+$ mice is slightly less severe (never reaching the scores of 4 and 5) compared to the IFNGR1-KO mice and tends to revert to intensive classical EAE (score of 2.5 and higher) after 2-4 days.

The immune cells infiltrating the CNS are not dominated by neutrophils and the moDCs are present in much higher proportion compared to the IFNGR1-KO controls (Figure 15b). No changes in proportions of any of the cell populations examined so far have been found. Cell-specific ablation of IFNγR on neutrophils and subset of myeloid cells seems to be sufficient to drive atypical EAE.

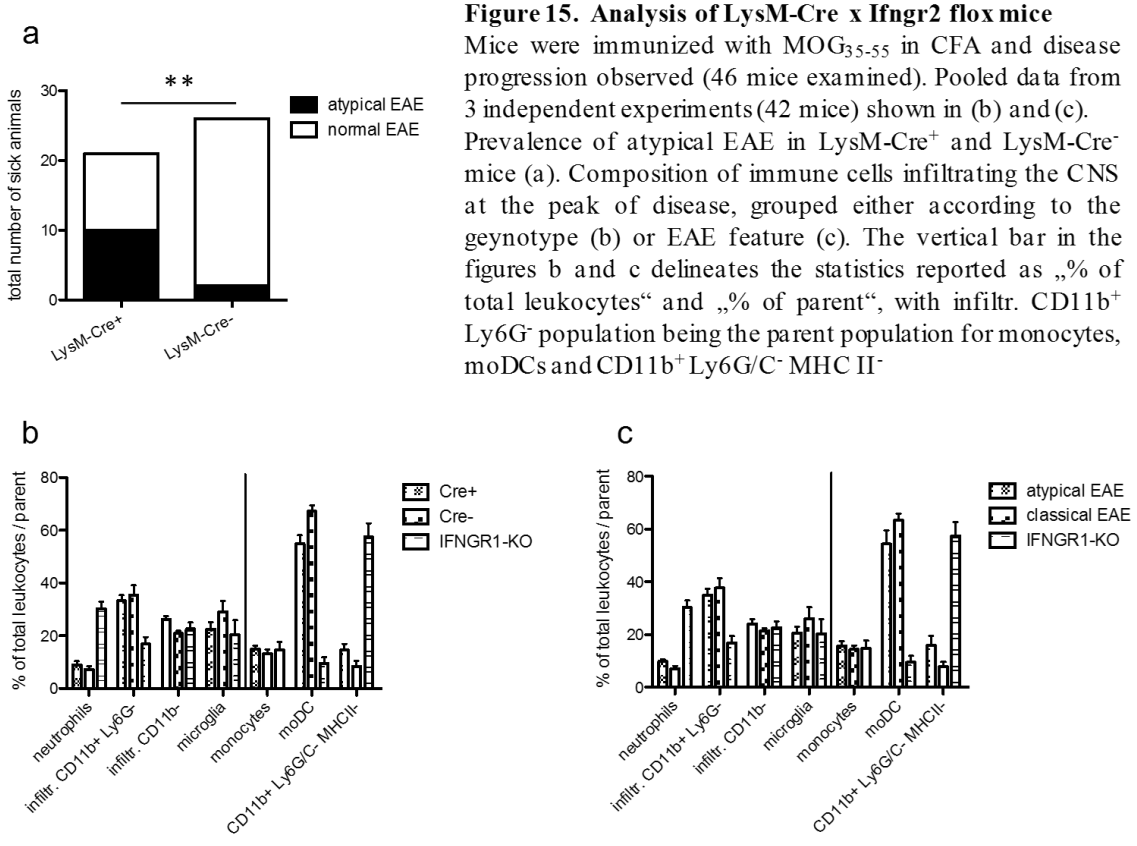

Figure 15. Analysis of LysM-Cre x Ifngr2 flox mice
Mice were immunized with MOG_{35-55} in CFA and disease progression observed (46 mice examined). Pooled data from 3 independent experiments (42 mice) shown in (b) and (c). Prevalence of atypical EAE in LysM-Cre$^+$ and LysM-Cre$^-$ mice (a). Composition of immune cells infiltrating the CNS at the peak of disease, grouped either according to the geynotype (b) or EAE feature (c). The vertical bar in the figures b and c delineates the statistics reported as „% of total leukocytes" and „% of parent", with infiltr. CD11b$^+$ Ly6G$^-$ population being the parent population for monocytes, moDCs and CD11b$^+$ Ly6G/C$^-$ MHC II$^-$

Since multiple reports showed vastly different activity of Cre recombinase driven by the lysozyme M promoter, we also investigated whether a different extent of Cre activity in different cell populations of Cre^+ mice could account for the occurrence of atypical EAE.

We assessed the efficiency of Cre-mediated recombination by flow cytometric analysis of pSTAT1. To achieve maximum reproducibility of the analysis and avoid bias in gating, an Overton subtraction algorithm was used to calculate the proportion of cells that have responded to IFNγ stimulation *ex vivo* (Overton, 1988) Depending on the shape of the histogram (unimodal vs. bimodal), the algorithm reports statistic that cannot be used as a measure of efficiency of Cre-mediated recombination and is different to what would be calculated by other methods. For instance, the efficiency of Cre-mediated recombination in neutrophils has been reported to be almost 100%, and this is what one also captures by eye when looking at the pSTAT1 staining histograms of appropriate mice (Figure 17). The Overton subtraction algorithm, however, reports 20% of neutrophils still being able to respond to IFNγ, which is most likely an overestimation and is due to the different frequency of cells away from the mode of the distribution. At the same time, monocytes in Cre^- mice show almost 100% responsiveness to IFNγ stimulation, as one would expect. The algorithm reports, however, only 70% of responding cells, which is an underestimation and is due to the overlap of pSTAT1 histograms of stimulated and unstimulated cells (Figure 17). Taken together, the values reported by the Overton subtraction algorithm should be used only for comparison among each other and should not be used to infer efficiencies of Cre-mediated recombination intended for comparison to readouts of other methods (reporter gene expression, qRT-PCR, Western Blot or similar). That said, neutrophils show the most extensive Cre-mediated gene rearrangement, compared to other myeloid populations showing a lower and more varying extent (Figure 16a). The interpretation of the data is made complicated by the fact that MHC II expression is used as a defining surface marker for describing certain subset of cells (monocyte-derived DCs, for instance) but the surface expression of the molecule only happens in the response to the IFNγ stimulation. It is, therefore, conceivable that the investigation of IFNγ responsiveness of moDCs would be biased against the cells undergoing the Cre-mediated recombination, since they would most likely not be included in the defining gate. Nevertheless, there seems to be no difference between the Cre^+ mice showing atypical or classical EAE in terms of

Cre-mediated recombination among the different cell subsets examined (Figure 16b). In other words, the observation of a LysM-Cre$^+$ mouse having atypical EAE does not correlate to a different efficiency of Cre-mediated recombination in examined subsets of myeloid cells.

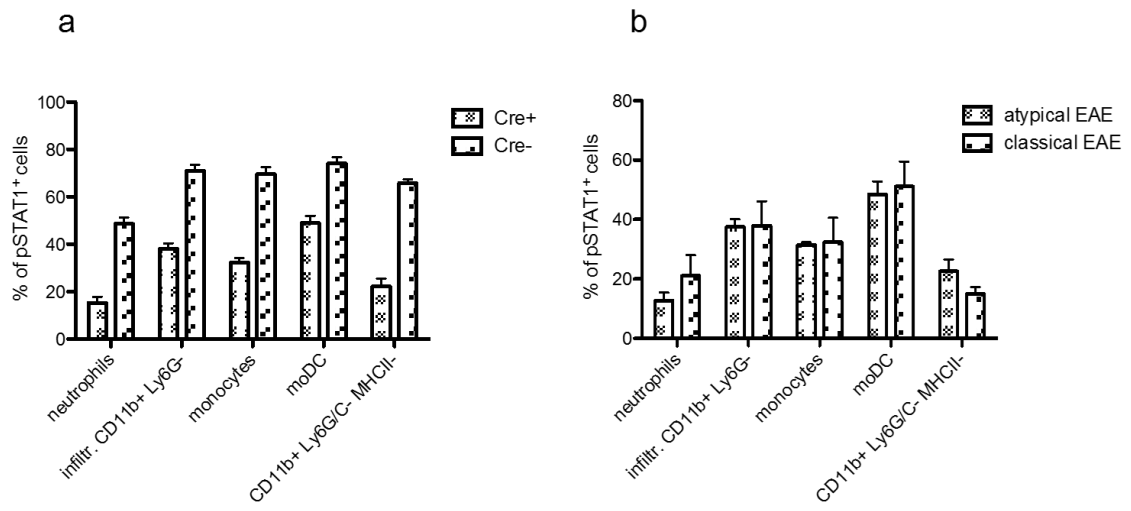

Figure 16. Analysis of the efficiency of Cre-mediated *Ifngr2* disruption accorss multiple cell types
Mice were immunized with MOG_{35-55} in CFA and inflamed CNS collected at the peak of disease. Isolated leukocytes were stimulated and prepared according to the Methods section for intranuclear staining of phosphorilated STAT1. Overton subtraction algorithm was used to calculate the proportion of pSTAT1$^+$ cells based on the difference between overlayed histograms of stimulated vs non-stimulated sample. Data from 1 experiment with 5 mice per group is shown.
Proportion of pSTAT1$^+$ cells among the designated cell populations (a). Proportion of pSTAT1$^+$ cells from LysM-Cre$^+$ mice grouped according to the EAE type displayed.

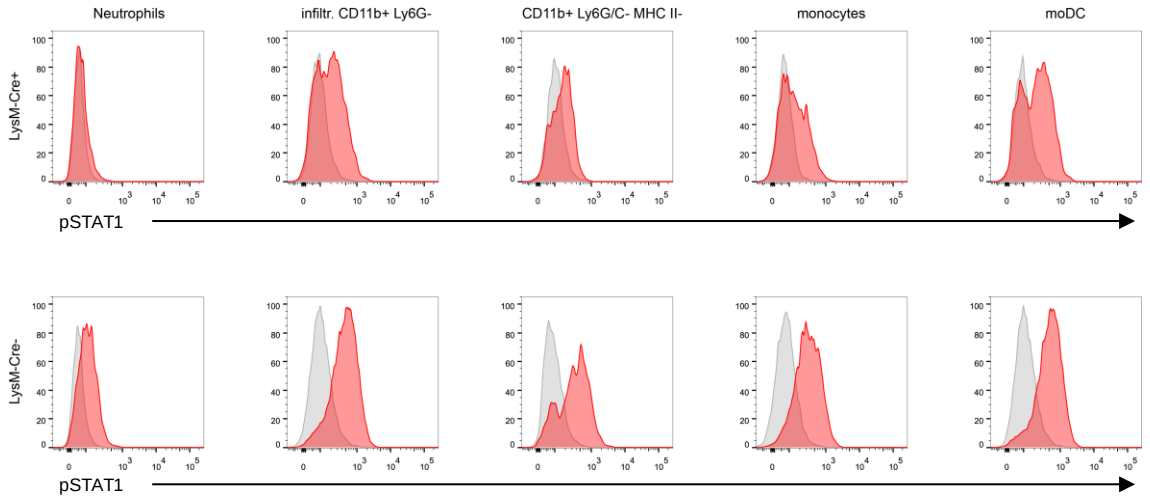

Figure 17. Analysis of the efficiency of Cre-mediated *Ifngr2* disruption accorss multiple cell types
Representative figures from the dataset in Figure 16.

Are neutrophils the main drivers of atypical EAE?

So far the role of neutrophils in atypical EAE has not been established. The impressive amount of neutrophils found in CNS lesions of IFNγ-KO and IFNγR1-KO mice correlates with the occurrence of atypical EAE, but some have suggested that there is no causal link among the two (Kroenke et al., 2010; Lee et al., 2012). There are reports claiming that neutrophils are non-redundant for EAE in general, and their depletion leads to complete EAE resistance (Kroenke et al., 2010; McColl et al., 1998). However, McColl et al. report that this requirement is not so strict in passive EAE, where neutrophil depletion results only in milder EAE course. The very early work on mice deficient in IFNγ signaling almost never reported occurrence of atypical EAE. Instead, there has been a consensus that EAE in these mice is exacerbated (Chu et al., 2000; Ferber et al., 1996). This leaves a possibility that atypical EAE was potentially misinterpreted as exacerbated form of classical EAE. Therefore, it is worthy to reevaluate the work of McColl et al. and see if neutrophil depletion during passive EAE results in modulation of atypical EAE, which might shed light on the role neutrophils play, if any.

We have set up the passive EAE experiment in a way that was previously reported to induce atypical EAE - transferring IFNγ-deficient cells into WT recipients. Neutrophils were depleted by repeated administration of the anti-Ly6G antibody (1A8 clone), starting 1 day before the adoptive transfer and administering the same dose (200 μg per mouse) every fourth day. Depleting neutrophils in mice either not transferred with encephalitogenic cells or transferred but inspected prior to the disease onset has proven to be effective (complete absence of neutrophils, Figure 18b and 18c). However, by the time mice started to develop EAE, depletion was not effective anymore and there was no difference in the proportion of neutrophils among anti-Ly6G- and isotype-treated group (Figure 19b). A potential caveat is the fact that neutrophils in anti-Ly6G treated mice stained less bright for Ly6G (now with Gr-1 isotype, Figure 16a) and evaluating depletion based on gates set on isotype-treated mice would be misleading and would result in a report showing complete absence of neutrophils. However, the light scatter profile suggests that these cells are, most likely, indeed neutrophils. The passive EAE was fully penetrant and resulted in severe EAE (Figure 18a). The composition of immune cells infiltrating the CNS was indistinguishable between the two groups (Figure 19c). However, there was no consistent occurrence of atypical EAE in any of the groups and, therefore, no

conclusions about the role of neutrophils in atypical EAE could be drawn. In the future, this experiment needs to be repeated using a different approach.

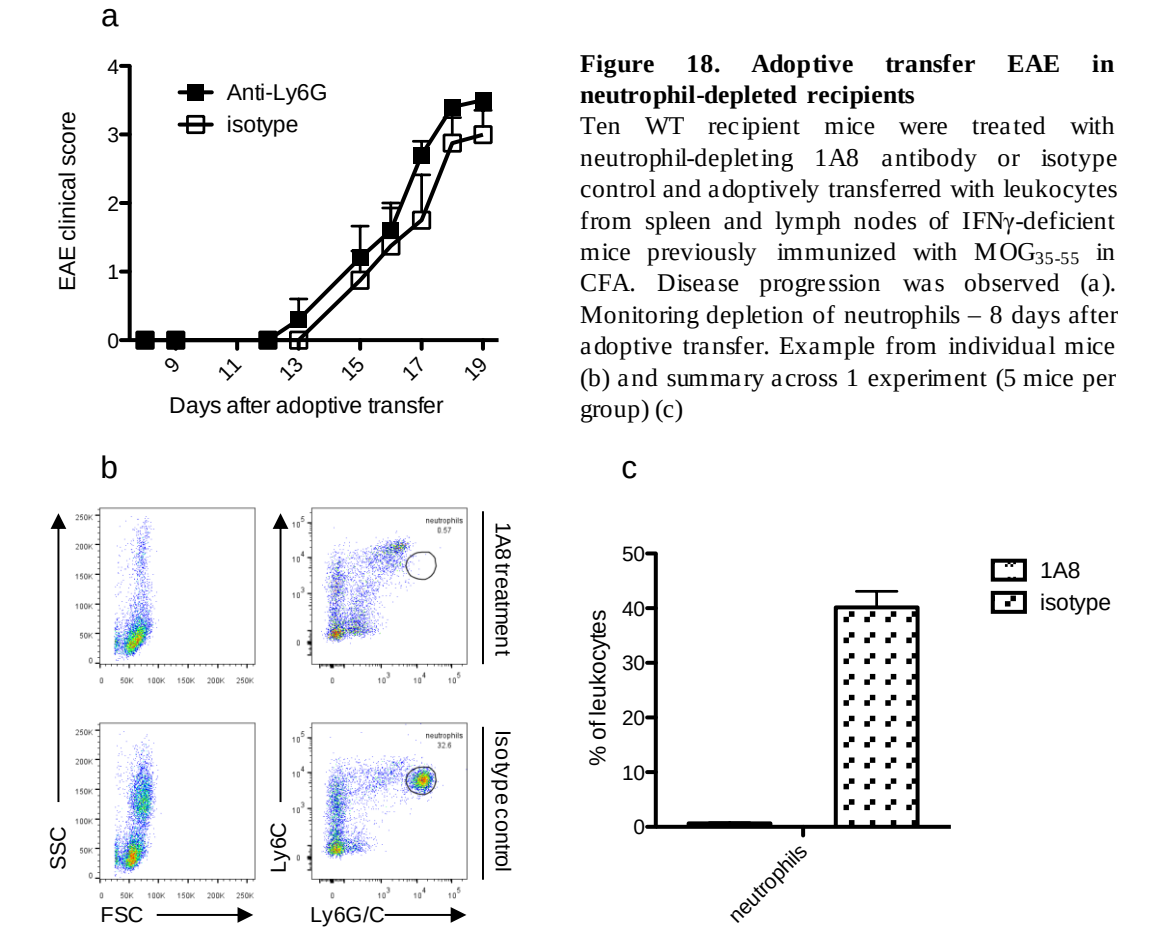

Figure 18. Adoptive transfer EAE in neutrophil-depleted recipients
Ten WT recipient mice were treated with neutrophil-depleting 1A8 antibody or isotype control and adoptively transferred with leukocytes from spleen and lymph nodes of IFNγ-deficient mice previously immunized with MOG_{35-55} in CFA. Disease progression was observed (a). Monitoring depletion of neutrophils – 8 days after adoptive transfer. Example from individual mice (b) and summary across 1 experiment (5 mice per group) (c)

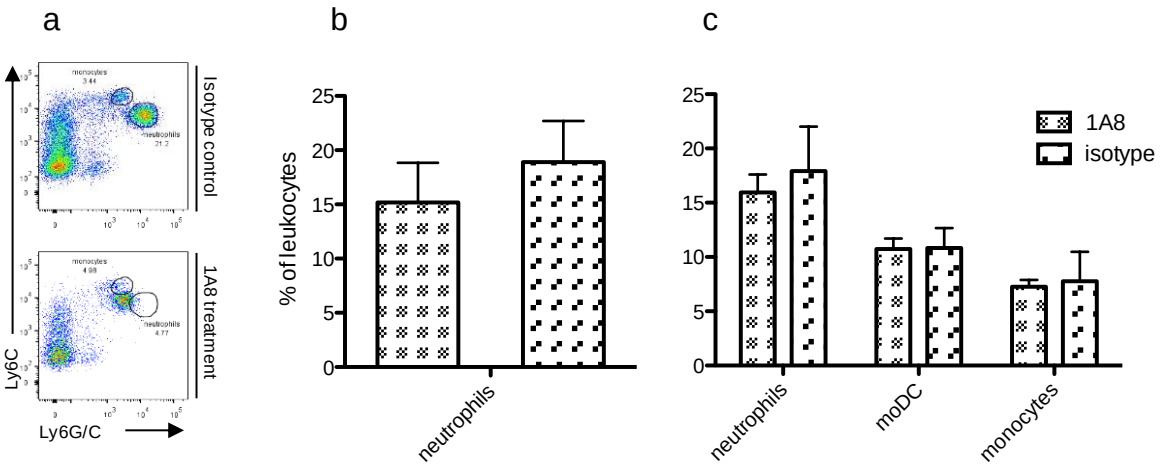

Figure 19. Adoptive transfer EAE in neutrophil-depleted recipients
Monitoring depletion of neutrophils from peripheral blood at the onset of EAE. (a). Proportion of neutrophils in peripheral blood of mice treated with the depleting antibody (clone 1A8) or isotype control at the disease onset (b). Composition of immune cells infiltrating the CNS at the peak of disease (c).

Discussion

The atypical EAE observed in mice deficient in IFNγ signaling is not unique to that particular experimental system - it has also been reported in various other systems, where a certain combination of mouse strain and immunizing agent was used (Greer et al., 1996; Muller et al., 2000). But, the genetic system of IFNγ deficiency likely provides the most precisely defined system, with the fewest amount of variables that affect the outcome. This is convenient since it clearly suggests the starting point for dissecting the complex biological system and asking a question - what is the causal link between the IFNγ deficiency and occurrence of atypical EAE? This is a somewhat simplified view and is not without caveats. The obvious one is - how general this observation is? In our hands, deficiency of IFNγ signaling has consistently resulted in occurrence of atypical EAE. However, as with EAE in general, it is not clear why animals of the "same" genetic makeup and maintained in the "same" environment sometimes succumb to the atypical disease while sometimes they do not. The older reports that sought to investigate the impact of IFNγ deficiency on autoimmune inflammation of the CNS reported exacerbated EAE, rather than atypical one (Chu et al., 2000; Ferber et al., 1996). One could argue that the more exacerbated EAE could have been a misclassified case of atypical EAE, since the atypical EAE has not been well documented back then. However, some of the more recent work also reports exclusively classical EAE in IFNγ-deficient mice (Wang, 2006). In my personal view, investigating atypical EAE on its own is valuable since it addresses one of the features that is often subject of criticism of EAE as a model for MS, in that it shows an unexplained bias of inflammation in the spinal cord over the brain, which is not the case in human disorders. Since atypical EAE shows no bias of that kind, it might provide additional insight into the basic mechanisms of autoimmune inflammation of the CNS.

IFNγ plays many different roles in the immune system (Boehm et al., 1997; Schroder et al., 2004). It should not come as a surprise then, that it was also implicated in autoimmune inflammation of the CNS. Big body of circumstantial evidence exists that links IFNγ and inflammation in MS and EAE. Many of the hallmarks of autoimmune inflammation are consistent with the reported effects of IFNγ - increased MHC II expression, macrophage activation, increased expression of adhesion molecules on the BBB (Olsson, 1992) and reactive gliosis. It is also detected in active MS lesions (Traugott and Lebon, 1988a, 1988b) and increased numbers of IFNγ-secreted T cells were detected in the CSF of MS

patients (Olsson et al., 1990). In EAE, the disease can be elicited in naive mice by adoptive transfer of IFNγ-producing T cells and animals with severe EAE show higher amounts of IFNγ mRNA in the CNS compared to the mice with mild EAE (Renno et al., 1994; Zamvil and Steinman, 1990). There have been only few clinical trials performed aimed at directly investigating the role of IFNγ in MS. In 1987 Panitch et al. reported that 7 out of 18 RRMS patients receiving IFNγ i.v. experienced exacerbations during the treatment period (Panitch et al., 1987), suggesting a disease promoting role for IFNγ. Similar conclusions were reached by another trial performed by Skurkovich et al., where they reported that treatment with antibodies against IFNγ was beneficial in secondary progressive MS (Skurkovich et al., 2001). In EAE, there are reports suggesting both disease-limiting and disease-promoting role for IFNγ. However, the work that involved either physiologically relevant interventions or utilized genetic deficiency systems mostly reported a disease limiting role, which is in contrast to conclusions drawn from human medical trials (Sanvito et al., 2010).

We have shown that disruption of IFNγ signaling in mice susceptible to EAE induction leads to the occurrence of atypical EAE which is characterized by dominant tissue damage in the cerebellum, rather than the spinal cord. IL17A is dispensable for this process. The composition of immune cells infiltrating the CNS does not necessarily correlate with the type of EAE and, hence, the dominant site of CNS tissue damage. There have been some reports implicating chemokines as the decisive factor determining the site of dominant CNS tissue damage (Abromson-Leeman et al., 2004; Tran et al., 2000). The conclusions drawn by the authors, if appropriate and applicable across different mouse strains, would suggest that the cell type responding to the lack of IFNγ signaling by producing a different set of chemokines would have to be present at the site of inflammation since, by definition, chemokines act only on short distances. The bone marrow chimera experiments we performed suggested that it is the inability of the invaded tissue to respond to the IFNγ that allows for the occurrence of atypical EAE, much more so than disrupted signaling in the invading cells. The same concept was also put forward by other authors (Lee et al., 2012; Lees et al., 2008). Taken together, it seemed reasonable to disrupt IFNγ signaling in the cells of the invaded tissue first.

We have examined all the cell types present in the CNS and found that, when examined separately, disruption of IFNγ signaling in them is insufficient to induce atypical EAE. Notably, some of the Cre strains used had even broader

activity and have targeted disruption of IFNγ signaling to additional cell types then the ones they were primarily intended for. For instance, it has been reported and we have also observed that the VE-Cadherin-Cre mouse strain shows recombinase activity in roughly 50% of circulating leukocytes, in addition to the vascular endothelium. This suggests that even broader disruption of IFNγ signaling than initially expected was still insufficient to drive atypical EAE.

A special case depicts the nestin-Cre x *Ifngr2 flox* strain we have created and examined in several experiments. In 2 independent experiments these mice showed atypical EAE. However, the occurrence of it did not correlate with the genotype as there was no statistical difference between the 2 groups, with or without the Cre-recombinase present. When the total of 6 independent experiments was examined together, these 2 "unusual" experiments did not affect the outcome of analysis - expectedly, there was no statistical significance in the difference observed between the two groups. Previous publications suggested that nestin-Cre transgene construct shows a tendency to generate germline deletion (Bates et al., 1999; Buchholz et al., 2000; Dubois et al., 2006). There are multiple nestin-Cre strains available. Although this particular aspect was not extensively investigated for the strain we used, it was clearly shown by the strain creator that the Cre recombinase was active in the CNS during embryonic development. Although recombinase activity outside desired tissue is unfortunate and generally compounds the interpretation of results, in our situation this was not the case. As long as the CNS was the main site of Cre recombinase activity (which is the point seemingly confirmed by a multitude of publications) our conclusions about disrupted IFNγ signaling in the CNS and atypical EAE are valid. Unfortunately, we were not able to investigate the expression patterns of our strain in greater detail. Following an isolated hygienic incident, the majority of the mice of this strain were sacrificed and the whole colony was re-established from a single mouse carrying the nestin-Cre transgene. Obviously, this presents a serious bottleneck in the population genetics and it was later confirmed that one of the founding animals had undergone a germline deletion of the floxed *Ifngr2* gene, resulting in mice fully deficient in *Ifngr2* gene regardless of the presence of the Cre transgene. We have every reason to suspect that similar events were also taking place in the original mouse colony, but with low frequency and masked to some extent since the offspring of multiple breeding pairs was randomly mixed. In our view, this represents the most plausible explanation for the results obtained from two aberrant experiments and

is the reason why the extent of Cre-mediated recombination at the *Ifngr2* locus was so far not independently confirmed by us. As soon as a proper colony status is re-established, this will be performed. Until then, the total number of nestin-Cre mice examined by us and previous published findings leave little doubt in the conclusions we have drawn.

In an attempt to reconcile the reports suggesting a role of chemokines and considering the possibility that our bone marrow chimera experiments were misleading, we decided to disrupt IFNγ signaling in other cell types invading the CNS during autoimmune inflammation. Our experiments with bone marrow chimeras suggested a more dominant role of IFNγ signaling in radiation-resistant cell type(s) than in radiation-sensitive one(s). However, it is possible that the cytokine storm following the irradiation process and incomplete engraftment of the transferred bone marrow masked the true nature of the initiating event in atypical EAE, leading us to too narrow conclusions.

We were able to show that disrupted signaling in T cells is insufficient to promote atypical EAE. The same was the case for mice with CD11c promoter-driven Cre expression, but it was later shown that this tool fails short in disrupting the IFNγ signaling in $CD11c^+$ cells of the inflamed brain, probably due to different kinetics of CD11c and IFNγR2 regulation (Andrew Croxford, unpublished observations). Surprisingly, we found that lysozyme M (LysM) promoter-driven Cre expression and resulting disruption of IFNγ signaling in neutrophils and subset of myeloid cells is sufficient to induce atypical EAE. The genetic system where the LysM promoter drives Cre expression allows for almost complete IFNγ signaling disruption in neutrophils and, most probably, macrophages (Clausen et al., 1999; Jakubzick et al., 2008; Ye et al., 2003), while the remaining cell types activating the locus are targeted to a various extent. In mice with autoimmune inflammation of the CNS, the only other "remaining cell type" present at the site of inflammation is monocyte-derived dendritic cells (moDCs).

To best of our knowledge, the deficiency of IFNγ signaling in moDCs has not yet been reported to play a role in atypical EAE. On the other hand, the moDCs have been implicated as important cell type in EAE in general (Mildner et al., 2009). Their putative role in allowing for the occurrence of atypical EAE is currently the matter of intense work by the author. On the other hand, neutrophils have been investigated by us and others as a potential cell type that plays a critical role in atypical EAE. While some groups (Abromson-Leeman et al., 2004; Kroenke et al., 2010; Lees et al., 2008; Wensky et al., 2005) reported that

neutrophils are associated with CNS lesions giving rise to atypical EAE, there is a number of reports showing that an extensive amount of neutrophils at the site of CNS inflammation does not necessarily induce atypical EAE (Kroenke et al., 2010; Lee et al., 2012; Tran et al., 2000). McColl et al. and Kroenke et al. reported that depleting neutrophils renders mice resistant to EAE induction. However, since McColl et al. used an anti-Gr-1 depleting antibody which is not specific for neutrophils, it is likely that they have also depleted some additional myeloid cell types. Kroenke et al. claimed successful neutrophil depletion and sparing of other leukocyte subsets using the CXCR2 anti-serum. However, since those mice were resistant to EAE induction, it was not possible to examine the role of neutrophils in atypical EAE. However, McColl et al reported that adoptive transfer EAE is less dependent on neutrophils and upon their depletion, mice show only slightly lower disease severity. This opens a possibility to investigate their role during atypical EAE. Our attempts so far have failed to provide concrete clues as we have been unable to maintain the depletion status of neutrophils once the mice started developing EAE symptoms. The experiments are underway to tackle this problem using a different approach.

Regarding the potential mechanism of action, Chu et al. suggested that the exacerbated EAE seen in IFNγ-deficient mice might be caused by the excessive amounts of CD4 T cells infiltrating the CNS as a consequence of their more intense proliferation and increased resistance to apoptosis in the absence of IFNγ signaling (Chu et al., 2000). These are interesting conclusions since the work of others suggests that in the absence of IFNγ-induced phosphorylation, STAT1 drives constitutive transcription of genes such as caspases (Kumar et al., 1997; Lee et al., 2000), which would act opposite to the observed findings. With regards to our data, we never noticed 10-16 fold increase in number of CD4 T cells during atypical EAE in IFNγ deficient mice, as reported by Chu et al. In fact, the proportions of CD4 T cells mostly remained the same, regardless of the type of EAE. So, the sheer number of infiltrating CD4 T cells doesn't seem to be required for the development of atypical EAE.

Tran et al. reported that IFNγ shapes the immune invasion of the CNS via regulation of chemokines (Tran et al., 2000). They found that IFNγ deficiency in mouse strains normally resistant to MBP-induced EAE renders them susceptible to disease induction and results in development of rapidly progressing lethal disease. They report widespread demyelination and disseminated leukocytic infiltration of the spinal cord and infiltrates dominated by Gr1+ neutrophils. They

found no expression of RANTES and MCP-1 but instead they reported elevated levels of MIP-2 and TCA-3. Lymph node cells from IFNγ-deficient mice proliferated in response to MBP, whereas the ones from IFNγ-sufficient mice did not. Overall, they conclude that IFNγ exerts a regulatory role in EAE, acting on proliferation of T cells and directing chemokine production. Our data shows that in mice normally conducive to active EAE induction there is no requirement for an increased CD4 T cell proliferation in atypical EAE resulting from disruption of IFNγ signaling. The implied role of chemokines in regulating the composition of immune cells is plausible and has been also suggested by others (Abromson-Leeman et al., 2004). However, given that we have disrupted IFNγ signaling in almost every cell type present at the site of inflammation without inducing atypical EAE, it is unclear which cell type would be the source of the particular chemokines that are upregulated as a consequence of the absence of IFNγ. In accordance to our findings, neutrophils might play this role, since they have been shown to both produce and respond to MIP-2 (Scapini et al., 2000).

Similar findings were reported by Krakowski et al., by using another strain resistant to MBP-induced EAE, namely BALB/c mice, and showed that targeted disruption of the IFNγ gene (GKO mice) converted them to a susceptible phenotype with as many as 17 out of 24 of IFNγ-deficient mice developing EAE, compared to mere 1 out of 16 in case of IFNγ-sufficient mice. They found meningeal and perivascular infiltrates in both spinal cord and brain, and have not reported any excessive accumulation of neutrophils at the site of inflammation (Krakowski and Owens, 1996).

Wensky et al. reported that the occurrence of atypical EAE depends solely on the capacity of CNS-invading T cells to produce IFNγ. They used a mouse strain that spontaneously develops a severe, nonclassical form of EAE with 100% incidence. The distinct clinical phenotype was marked initially by a slight head tilt, progressing to a severe head tilt, spinning, or a rotatory motion. Classical EAE spontaneously occurred in MBP-specific TCR transgenic RAG-$1^{-/-}$ mice (referred to as T/R^{-}), whereas nonclassical EAE spontaneously occurred in T/R^{-} IFN$\gamma^{-/-}$ ($T/R^{-}\gamma^{-}$). Thus, the TCR recognized the same Ag (MBP) in both cases. The cellular infiltrates in nonclassical EAE were predominantly found in the brainstem and cerebellum, with very little inflammation in the spinal cord, which was in turn primarily affected in classical disease. Importantly, they also showed that, depending on the genetic makeup and priming conditions of the MBP-specific T cells, nonclassical disease can occur in the presence of an inflammatory

infiltrate with eosinophilic, neutrophilic, or monocytic characteristics (Wensky et al., 2005). In principle, their work also supports the notion that neutrophil-rich immune cell infiltrates are not necessary for the occurrence of atypical EAE. They speculate that T cells that cause atypical disease potentially express a different chemokine repertoire that allows for their preferential recruitment to a specific site of the CNS.

Stromnes et al. also report that the TCR specificity and cytokine repertoire of CD4 T cells are the main determinants of the type of EAE induced. They showed that the initiating events in both types of EAE are the same but the effector profile of the CD4 T cells effectually influences whether the brain or the spinal cord develop more severe inflammation. Although they put a strong emphasis on the nature of CD4 T cells, they show that the invaded tissue produces large amounts of IL17A and that neutralization of IL17A abolishes the capacity of CD4 T cells to invade the brain parenchyma specifically. With regards to the genes showing disproportionate increase in expression specifically in atypical EAE brains an upregulation consistent with enhanced local production of IL17A was revealed (Stromnes et al., 2008). Although this group makes a strong argument, our data shows that IL17A dependency is not unique across multiple strains, as Stromnes et al. used C3H mice in their studies. Our findings also in general reduce the importance of the cytokines produced by CD4 T cells as a determining factor for the outcome of EAE. Instead, it is more likely the tissue response to these cytokines that plays a decisive role and it is conceivable that various molecular stimuli (or lack thereof) could trigger the same or similar tissue response in terms of production of particular inflammatory intermediates. We think that this distinction, although minor, is relevant since Stromnes et al. speculated about the clinical relevance of their findings and the apparent role IL17A plays. Our results would clearly suggest otherwise.

When discussing potential mechanisms of action, it is worthy to mention the study of Rose et al., who reported an augmentation of EAE when a monoclonal antibody to ICAM-1 was administered after adoptive transfer of encephalitogenic cells. The clinical disease was more severe in the anti-ICAM-1 treated EAE mice and included prominent ataxia compared to the PBS-treated controls. Neuropathological evaluation demonstrated a distinct distribution of lesions in the anti-ICAM-1-treated mice which featured prominent demyelination and inflammation of the cerebellum, brainstem and cerebrum. These structures were minimally involved in the control mice (Rose et al., 1999). This report

emphasizes two important concepts. Rose et al. do not report extensive neutrophil infiltration of the inflamed CNS. Instead, they show immune infiltrates dominated by T cells. Their findings suggest once more that excessive neutrophilia is not required for the occurrence of atypical EAE. Additionally, they show that blockade of ICAM-1 is sufficient to induce atypical EAE. ICAM-1 is expressed by endothelial cells and APCs. While it has been shown that multiple cytokines can upregulate ICAM-1 on endothelial cells *in vitro* (IL-1, TNFα, IFNγ), Koide et al. reported that neutralization of IFNγ completely inhibits ICAM-1 expression on vascular endothelial cells following injection of LPS *in vivo* (Koide et al., 1997). This provides a link to our work, allowing to suggest a potential explanation of the mechanism. Unresponsiveness to IFNγ could lead to the absence of ICAM-1 upregulation which, in turn, could result in atypical EAE. However, assuming that IFNγ indeed is absolutely required for upregulation of ICAM-1 on vascular endothelium (Boehm et al., 1997), we have shown that targeted disruption of IFNγ signaling on endothelial cells is insufficient to drive atypical EAE, suggesting that blocking ICAM-1 and probably also expression of a few other adhesion molecules is not a mechanism of action allowing atypical EAE in our experimental system. Another cell type known to express ICAM-1 are APCs. It would be interesting to investigate whether disruption of IFNγ signaling in subset of myeloid cells in the LysM-Cre x Ifngr2 flox mice used in our work interferes with ICAM-1 expression and plays a role in the occurrence of atypical EAE. Additionally, the finding that IFNγ signaling on vascular endothelium is dispensable for efficient extravasation of leukocytes into the inflamed CNS presents an interesting starting observation for additional investigation.

Willenborg et al. reported that 129/Sv mice, normally resistant to the induction of EAE with MOG, become susceptible upon deletion of the gene coding for the ligand-binding chain of the IFNγ receptor (*Ifngr1,* IFNGR1-KO mice). These mice develop EAE with high morbidity and mortality. Importantly, splenocytes from sensitized IFNGRl-KO mice proliferated extensively when stimulated with MOG peptide in culture and produced high levels of IFNγ. In both active and passive EAE there was an extensive involvement of neutrophils in the CNS lesions of IFNGR1-KO mice. (Willenborg et al., 1996). A few important points need to be re-emphasized from their work. They neither report atypical EAE nor mention disease signs that we now routinely classify as atypical EAE. The inability to sense IFNγ translates into more severe EAE and extensive

neutrophil infiltration of the CNS. Splenocytes deficient in IFNγ receptor still produced high levels of IFNγ upon MOG stimulation *in vitro*. In principle, the work from Willenborg et al confirms once more that neutrophil-rich infiltrates in the CNS do not necessarily induce atypical EAE. However, neutrophilia seems to be a consistent companion of EAE in IFNGR1-KO mice, which perfectly matches our findings in IFNGR1-KO mice. They also clearly reveal a point which we believe is a mistaken argument that compounds a unified interpretation of some work in the field of atypical EAE: A lot of emphasis has been put on the ability of CD4 T cells to produce IFNγ. However, given the fact that CD4 T cells are the main producers of IFNγ during EAE, it seems obvious that their inability to produce IFNγ most likely means that there is less IFNγ in the whole system. This implies that inability of CD4 T cells to produce IFNγ is somewhat equivalent to the inability of target tissues or cells to sense the cytokine - simply because there is not sufficient amount around.

This concept was also supported by the work of Lees et al., who showed, by using an adoptive transfer system, that the ability of the CNS to sense pathogenic T cell-derived IFNγ during EAE initiation determines the sites of CNS pathogenesis. Transfer of WT Th1 cells into IFNγ receptor-deficient mice resulted in pathogenic invasion of the brain stem and cerebellum with clinical symptoms of atypical EAE, which were identical to the disease observed after transfer of IFNγ-deficient T cells to WT hosts. Inflammation of the spinal cord associated with classical EAE was abrogated in both IFNγ-deficient systems. Cotransfer of CNS antigen-specific WT Th1 cells with IFNγ-deficient T cells was sufficient to restore spinal cord invasion and block cerebellar and brain stem invasion (Lees et al., 2008). Their data demonstrates that IFNγ interacts with either the CNS cells or other non-T cells capable of sensing IFNγ during the initiation phase of EAE and that the this interaction likely influences the subsequent steps of neuroinflammation and pathogenesis.

Abromson-Leeman et al. reported two distinct clinical phenotypes of EAE in BALB/c GKO mice immunized with encephalitogenic peptides of MBP. Conventional disease, characterized by ascending weakness and paralysis, occurred with greater frequency after immunizing with a peptide comprising MBP residues 59 to 76. Axial-rotatory disease, characterized by uncontrolled axial rotation, occurred with greater frequency in mice immunized with a peptide corresponding to exon 2 of the full length 21.5-kDa protein. The conventional disease was characterized by inflammation and demyelination primarily in

spinal cord, whereas axial-rotatory disease involved inflammation and demyelination of lateral medullary areas of brain. Both types had infiltrates in which neutrophils were a predominating component. By isolating T cells and transferring the disease to naive recipients, they showed that the type of disease was determined entirely by the nature of inducing T cell type. Most intriguingly, they showed, using CXCR2 knockout recipients which are unable to recruit neutrophils to inflammatory sites, that among the two different cell lines derived from GKO mice, one was critically dependant on neutrophils to effect the disease (3A clone) while the other was not (X2.502 clone) (Abromson-Leeman et al., 2004). In a continuation of their work, they showed that neither of the two GKO-derived cell lines (now with slightly different nomenclature suggestive of derivatives/subclones of the original 3A and X2.502 clones) relied on IL17A to effect the disease (Abromson-Leeman et al., 2007). The authors once more show that composition of cells infiltrating the CNS in atypical EAE is not a determinant of ensuing clinical features. Also, they report an interesting observation, where an encephalitogenic T cell clone shows upregulation of a set of genes normally associated with Th17 effector phenotype but does not depend on IL17A itself to realize its encephalitogenic potential. This observation could, at least in theory, explain why we see no dependence on IL17A, while Th17 effector cells have been shown to be indispensable in some previous reports. Nevertheless, Stromnes et al. have clearly shown dependency of atypical EAE on IL17A, while Kroenke et al. reported dependency on IL17RA. To explain the difference between IL17A and IL17RA dependency is rather straightforward based on existing knowledge, since the IL17RA is needed for both IL17A and IL17F signaling. Thus, the relevant cytokine might be IL17F. So far we have not explicitly examined the potential role of IL17F in our experimental system. Also, we have not recognized the unifying aspect of our work and the work of Stromnes et al. in terms of IL17A relevance, if one exists at all. Unfortunately, the difference between our and their experimental setup is rather significant (passive EAE in C3H mice vs. active EAE in C57Bl/6) and this, unfortunately, might just be enough to not allow a direct comparison of conclusions. Hopefully, the continuation of our work might reveal some additional relevant comparison points.

In summary, taking into account all the correlated and non-correlated findings discussed so far, it is very likely that atypical EAE, as it is defined at the moment, is only an observable manifestation of multiple different neuroinflammatory pathways that preferentially inflict tissue damage to the

brain stem and cerebellum, rather than the spinal cord. The underlying mechanism of action is most probably not the same in all the different models presented. Despite that, they are all valuable tools to study a "different kind" of neuroimmune inflammation in rodents and provide a chance to gain additional insight into the immune mechanisms in effect during autoimmune inflammation of the CNS.

Conclusion and future work

Our work shows that disrupted IFNγ signaling in neutrophils and a subset of myeloid cells is sufficient to drive atypical EAE. The composition of the immune cells infiltrating the CNS is not a determinant of the ensuing clinical manifestation of EAE. The atypical EAE in the context of IFNγ deficiency is not dependent on IL17A nor is mediated by disrupted IFNγ signaling in microglia, as was suggested by some authors. Furthermore, absence of IFNγ signaling on vascular endothelium and presumed changes in the expression pattern of its adhesion molecules that result from it have no impact on the capacity of circulating leukocytes to extravasate and reach the CNS during neuroinflammation. Additionally, we were able to show that inability of CNS tissue to respond to IFNγ stimulation during neuroinflammation does not noticeably influence the extent of tissue damage inflicted by the infiltrating immune cells.

The finding that the mouse strain with disrupted IFNγ signaling in cells that have activated the lysozyme M promoter does not allow for a precise conclusion on which cell type plays a critical role in allowing for the occurrence of atypical EAE since multiple different cell types are affected - neutrophils and moDCs being the most notable ones. To address this in greater detail, we plan to deplete neutrophils from peripheral blood of IFNγR-deficient mice and adoptively transfer WT T cells from a mouse previously immunized with MOG_{35-55} in CFA. This adoptive transfer experiment would allow us to judge the requirement for neutrophils during adoptive transfer EAE. The possibility to induce atypical EAE in the mentioned experimental setup would suggest that moDCs play a crucial role in allowing for the occurrence of atypical EAE.

Additionally, crossing the *Ifngr2*^{tm1Hzi} mouse strain to CCR2-CreERt2 mouse strain would hopefully allow us to disrupt the IFNγ signaling in moDCs. Observing the clinical signs of EAE in this newly created strain will allow us to draw additional conclusions on the role of moDCs during atypical EAE and

show which cell type most likely plays a crucial role in allowing for the occurrence of atypical EAE.

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Contributions

The following people have contributed to the thesis:

- Barbara Ingold Heppner
  - Pathohistological analysis (H&E, LFB-PAS, Iba-1)
- Doron Merkler
  - Pathohistological analysis (H&E, LFB-PAS, Iba-1), immunohistochemistry (pSTAT1, Mac3, GFAP, NogoA, NeuN)
- Cornelia Halin Winter
  - Provided the VE-Cadherin-Cre mouse strain
- Steffen Jung
  - Provided CX3CR1-CreERt2 mouse strain
- Max Gassmann
  - Provided nestin-Cre mouse strain
- Werner Muller
  - Generated and provided Ifngr2 flox mouse strain

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If indeed early years in life are the most important formative years of a person, then my parents and my brother have every right to take pride in everything good I do since they were the surrounding that first nurtured my character. I am grateful for happy memories of my childhood and for always having a feeling of having a safe harbor over there, no matter where I was at that moment and no matter what happened.

The fact is, during the last 5 years, I have spent more time with my lab mates than with my family. Obviously, this was not only about pipetting ☺ Some of the people who were "the lab" when I was starting moved on and left Zurich. But some of them came back again and I am happy for that. Stefan and his dry ice "firecrackers", Gabor and his standing invitation for a beer and food, Maya, who sometimes knew better what I wanted than me… I am really happy to have these people around still! Of course, some never left… at least yet! I have cycled many kilometers (well, some, at least) and moved a lot of furniture around and, thinking about it, generally did many things with Flo… Not always do we share a common perspective on things ;), but we always, without exception, no matter what we did, got along really well without needing too many words. So well, in fact, that I suspect Sabi was sometimes jealous ☺ Sabi has also been a dear friend… She sometimes approves the way I do some things even less than Flo! But still, I could count on her whenever I needed to… Kathrin and Felix are my source of German grammar and expressions. They pretty much proof-read all the letters I wrote. Sabrina is very patient at teaching me Swiss German. She's nice

enough not to pun funny words in her sentences so I can pretend I understand "Thurgauerdütsch" perfectly!

I often say that one of the problems of doing a PhD is a serious lack of short-term rewards to keep you motivated and going. Well, that's only part of the story … People I've been and still am surrounded with make up for all what might be missing!

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Jan 2009 - present	**Doctoral candidate** *Institute of Experimental Immunology, University of Zurich, Switzerland* Research in basic immunology.
Mar 2011 – present	**Technology officer** *Flow cytometry core facility – Irchel, University of Zurich, Switzerland* Application and instrument support, user training, instrument operation.
Jan 2009 –Aug 2009	**Instructor and operator** *Flow cytometry core facility –Irchel, University of Zurich, Switzerland* Application and instrument support, user training, instrument operation.
Oct 2006 – Dec 2008	**Research and teaching assistant** *Department of physiology and immunology, Rijeka School of Medicine, Croatia* Research in basic immunology, mentoring students
Oct 2007 – Dec 2008	**Instrument operator** *Cell sorting service, Rijeka School of Medicine, Croatia* Instrument operation, application support

Additional training and certificates

Oct 2011	**International Cytometry Certification Examination** Successfully passed the examination and experience requirements and earned the title of **CERTIFIED CYTOMETRIST** according to ISAC and ICCS.
Jun 2011	**34th Annual Course in Flow Cytometry** *Center for Biomedical Engineering at University of New Mexico, Verity Software House, National Flow Cytometry Resource at the Los Alamos National Laboratory* University of New Mexico, NM, USA
Jun 2010	**33rd Annual Course in Flow Cytometry – Research Methods and Applications.** *Cytometry Educational Associates, National Flow Cytometry Resource and Verity Software House* Bowdoin college, Brunswick, ME, USA
Oct 2009	**Comprehensive Flow Cytometry Course.** *FloCyte Training Institute* San Raffaele hospital, Milano, Italy
Oct 2007	**BD FACSAria Operator Training** BD Biosciences Training Center, Erembodegem, Belgium

Personal skills and competences

Languages	Croatian: mother tongue English: proficient (C2) German: independent (B2) Italian: basic (A1)
Computer skills	Advanced multi-platform computer user (Windows, MacOS, Linux) Highly competent with common personal productivity software suites (Microsoft Office/Open Office, picture/audio/video editing and manipulation, etc.) Experienced in web design, HTML and client-side scripting Basic knowledge of server-side web programming. Basic knowledge of high-level programming languages (Python, Perl)
Additional skills	Cell-sorting and instrument operation in the BSL-3 environment

Publications

Codarri L, Gyülvészi G, **Tosevski V**, Hesske L, Fontana A, Magnenat L, et al. RORγt drives production of the cytokine GM-CSF in helper T cells, which is essential for the effector phase of autoimmune neuroinflammation. Nat. Immunol. 2011 Jun;12(6):560–7.